CHAPTER 3

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CEREAL PROTEINS

JEROLD A. BIETZ
Northern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
Peoria, Illinois

I. INTRODUCTION

Cereal endosperm storage proteins include primarily prolamins (the name derived from "proline" plus "amine," generally proteins soluble in aqueous solutions of alcohols) and glutelins (proteins insoluble under neutral, nonacidic, nonreducing conditions in water, saline solutions, and alcohol but soluble in solutions containing acid, alkali, detergents, denaturants, or disulfide reducing agents) (Osborne, 1907). Albumins (soluble in water) and globulins (soluble in saline solutions but insoluble in water) are also present, but to lesser extents. Recent reviews (Kasarda et al, 1976; Wall and Paulis, 1978; Wall, 1979; Bright and Shewry, 1983; Payne and Rhodes, 1983; Shewry et al, 1984) summarize current knowledge of the origins, structures, properties, and relationships of these proteins, which are of great functional and nutritional importance.

Analysis of cereal proteins may be extremely difficult. Cereal proteins are very heterogeneous, often being products of large multigene families. Their solubilities are also atypical of most other plant or animal proteins, resulting from their unusual amino acid compositions (generally rich in glutamine, proline, and hydrophobic amino acids and deficient in acidic and basic residues). Thus, analytical methods for other proteins may not succeed with cereal proteins. Finally, many cereal proteins interact noncovalently with endosperm constituents such as lipids and carbohydrates, and associate, either noncovalently through hydrogen or hydrophobic bonds or covalently through disulfides, with each other to form high-molecular-weight (HMW) complexes.

In spite of these difficulties, several analytical methods permit isolation and characterization of cereal endosperm proteins. After initial demonstrations (Jones et al, 1959) showed that cereal proteins could be separated by moving boundary electrophoresis, methods were developed using porous starch, polyacrylamide, or agarose gel matrixes (for reviews see Wrigley, 1977; Bietz, 1979; Wrigley et al, 1982) to separate proteins in three ways. In starch gel electrophoresis or polyacrylamide gel electrophoresis (PAGE), proteins are

separated by both size and charge since, at equilibrium, protein size affects diffusion through the matrix and ionizable side chains determine net charge (Woychik et al, 1961; Chrambach and Rodbard, 1971). In isoelectric focusing, however, the effect of size on separation is nearly eliminated, since proteins migrate to and stop at positions corresponding to their isoelectric points (Wilson, 1984). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins complex to the ionic detergent sodium dodecyl sulfate (SDS), which masks native charge differences, and migrate in an electric field almost totally on the basis of molecular size (Bietz and Wall, 1972; Paulis et al, 1975).

Chromatography is also useful for isolation and characterization of cereal proteins. Gel filtration (also called gel permeation, molecular exclusion, or size-exclusion chromatography) separates proteins primarily by size (see Bietz, 1979), whereas ion-exchange columns fractionate proteins by charge differences due to ionizable side chains (Huebner and Wall, 1966). More recently, hydrophobic interaction chromatography (HIC) has been used to fractionate cereal proteins, based on differing surface hydrophobicities, by interaction between proteins and porous hydrophobic column matrixes (Popineau and Godon, 1978; Caldwell, 1979; Chung and Pomeranz, 1979). These electrophoretic and chromatographic techniques, although providing useful separations and information, have insufficient selectivity and resolution for most cereal protein samples.

In recent years, chromatographic methods providing superior separations have been developed. These methods, termed high-performance liquid chromatography (HPLC), basically represent improved instrumentation and columns. Chromatographic systems that are highly reliable, and small silica-based columns that can withstand relatively high pressures (as indicated by the original definition of HPLC as "high-*pressure* liquid chromatography") and flow rates, due to their small, uniform, and stable packings, have become available. These columns provide improved speed, sensitivity, resolution, reproducibility, and ease of use. However, the relatively small (generally 80–100 Å) pore size of normal silica in the columns used to separate low-molecular-weight (LMW) organic compounds does not permit penetration and optimal separation of proteins having molecular weights in excess of approximately 15,000.

More recently, silica-based HPLC columns suitable for HMW proteins have become available (Regnier and Gooding, 1980; Hearn et al, 1982). Typical characteristics of reversed-phase (RP-) HPLC columns are summarized in Table I. These columns, generally designated "large-pore" or "wide-pore" columns, contain silica packings with pores 300 Å or larger, permitting the total silica surface to be accessible to and penetrated by polypeptides of molecular weights up to several hundred thousand. Various bonded phases may be covalently attached to silica silanol groups, resulting in RP-, ion-exchange (IE-), and size-exclusion (SE-) HPLC columns. The columns' ability to attach relatively high amounts of bonded phases ("carbon loading"), plus the deactivation of most remaining free silanols by "end-capping" (reaction with a small silylating reagent), gives these columns high capacity for proteins plus excellent recovery.

During the last few years, these new HPLC columns have been applied extensively to peptides and noncereal proteins. The heterogeneity of cereal

proteins, the problems associated with their isolation and characterization, and the stability of silica packings in solvents necessary to solubilize cereal proteins suggested to Bietz (1983) that HPLC might also be useful for cereal proteins. In addition, HIC, which, like RP-HPLC, separates proteins on the basis of surface hydrophobicity, had been shown to be a new, useful, and complementary method for fractionation of cereal proteins (Popineau and Godon, 1978; Caldwell, 1979; Chung and Pomeranz, 1979).

Bietz (1983) first reported the application of RP-HPLC to endosperm proteins of wheat and maize. Until then, RP-HPLC had been used primarily for large peptides or fairly simple (or standard) proteins and not for complex biological mixtures such as cereal endosperm proteins. Lookhart had previously tested RP-HPLC of wheat proteins (personal communication), but suitable columns were not then available; since stable columns giving high recoveries and no irreversible adsorption are now available, RP-HPLC of cereal proteins has succeeded where it failed before. Several studies have now determined optimal RP-HPLC conditions for various proteins and have applied RP-HPLC to significant problems. In addition, SE-HPLC of cereal proteins has become widely used, and initial studies involving IE-HPLC could be particularly valuable, since ion-exchange chromatography on cellulose columns provides excellent fractionation of cereal proteins. Many investigators now use HPLC to analyze cereal proteins and find that the method has numerous advantages.

This chapter reviews methods of HPLC analysis of cereal proteins. Optimal equipment and procedures are described in sufficient detail, along with practical information, to assist investigators in initiating HPLC separations. It then reviews specific examples of HPLC analysis of cereal proteins (not including amino acids, amino acid derivatives, or peptides derived from cereal proteins), describing the separations, their optimization, and specific applications. Finally, a brief discussion of future applications and utilization of HPLC in cereal protein chemistry is given.

II. HPLC METHODOLOGY

A. Apparatus

Excellent HPLC equipment is available from numerous manufacturers. This equipment can maintain accurate constant flow rates and generate the

TABLE I
Characteristics of Typical RP-HPLC Columns Suitable for Proteins

Component	Description
Support material	Uniform spherical silica
Support size	5–10 μm
Pore diameter	300–500 Å
Column dimensions	4–4.6 mm i.d., 5–30 cm long (analytical)
	10 mm i.d., 25 cm long (preparative)
Operating pressure	300–1,500 psi at 1.0 ml/min
Carbon loading	5–20% carbon by weight
Bonded phases	C18, C8, C4, C3, cyanopropyl, alkylphenyl, diphenyl, etc.
End-capping	Common

reproducible gradients essential for RP- and IE-HPLC. The major components of an HPLC system are described in the following paragraphs.

SOLVENT STORAGE

HPLC solvent reservoirs (and all other system components exposed to solvents) are composed only of stable materials such as glass, Teflon, and stainless steel. Provisions may be made to prevent evaporation from reservoirs to avoid changes in solvent composition and to maintain an inert atmosphere (e.g., helium) above the solvents.

PUMPS

HPLC solvent delivery systems must be capable of maintaining accurate and constant flow rates. Most pumps have maximum flow rates of 10–15 ml/min and minimum flow rates of about 0.1 ml/min; some newer systems (as for narrow-bore HPLC) also permit solvent delivery at lower flow rates. Solvent delivery systems are of two major types. In multiple-pump systems, each solvent is delivered by a separate pump and then mixed under pressure. Relative pump rates are regulated by a system controller in an integrated system or by a gradient former, which varies the voltages to pumps to control their output. In another type of solvent delivery system, two (or more) solvents are introduced, via variable solenoid valves under microprocessor control, to one pump. This latter apparatus is simpler and less expensive than multiple-pump systems and is acceptable for most applications.

PUMP CONTROL, SYSTEM CONTROLLER, OR GRADIENT FORMER

As indicated, in a multiple-pump system, a separate unit must vary the relative pump rates with time to form reproducible gradients. Gradient formers vary pump voltages with time, permitting linear and nonlinear gradients. In many chromatographs, however, a system controller forms gradients. It may also control an autosampler and detector and may acquire and process data through a computer. Advanced system controllers store raw chromatographic data for subsequent reintegration, replotting, and comparison.

INJECTORS

Some means of accurately introducing samples into the flowing mobile phase is necessary in any HPLC system. This is usually accomplished by introducing samples into loops, which then are placed in-line with the flowing mobile phase. Two types of injectors are used. In manual injectors, samples are loaded into loops with a syringe and introduced into the solvent stream by manually turning a valve. Such injectors are inexpensive, easy to maintain, and reliable. Automatic sample injectors, available from several manufacturers, are considerably more expensive (frequently $8,000–10,000) and more complex than manual injectors but are highly recommended; they permit unattended operation, freeing operator time for other duties, and are thus highly cost effective. Such injectors also significantly improve reproducibility by repeatedly injecting accurate sample volumes at reproducible intervals, thus avoiding operator error. Sample volume, number of injections, run time, and equilibration delay for each sample vial can also be individually programmed.

Sample volumes can range from 1 μl to 2 ml or more. Automatic sample identification may also be possible.

GUARD COLUMNS AND FILTERS

It is essential to place guard columns and/ or in-line filters between the injector and the column to promote maximum column life. Guard columns, available in many configurations and sizes, are basically small columns containing packing similar to that in the analytical column. They bind solutes that may irreversibly adsorb to and diminish the performance of packings, and they also serve as "final filters" by removing particulates introduced with the samples or arising from pumps or valves. In addition to a guard column (or in its place, if guard column packings comparable to those in analytical columns are not available), an in-line filter of about 0.5-μm porosity (e.g., that from Upchurch Scientific, Inc.) may be used; such filters do not adsorb harmful solutes but are generally adequate for removal of particulates.

COLUMN

The column, discussed in more detail below, is the heart of an HPLC system. Most analytical columns are housed in stainless steel cylinders, have an internal diameter of 4.0–4.6 mm, and are 250 mm in length; shorter columns (e.g., 75 mm) and semipreparative columns (e.g., 10 mm i.d.) are also available and are widely used. Glass-lined columns and plastic-walled cartridges are now also being introduced.

TEMPERATURE CONTROL

To improve reproducibility and resolution of HPLC separations, some means of maintaining a constant and elevated column temperature is necessary (Bietz and Cobb, 1985). Columns may fit into machined aluminum blocks, maintained at constant temperature by circulating water or by an electrical resistance heater. Alternatively, constant-temperature air ovens can be used, and "heat tape" types of temperature controllers are available.

DETECTORS

Ultraviolet (UV) absorbance detectors are usually used for HPLC analysis of proteins. In fixed-wavelength detectors, filters select the appropriate wavelengths (254 nm being suitable for most organic compounds); such detectors are relatively inexpensive, and their lamp life is frequently longer than that of variable-wavelength detectors. With variable wavelength detectors, however, any wavelength in the UV/ visible range from about 190 to 660 nm can be selected. Variable wavelength detectors cost more and may have decreased lamp lifetimes. They are highly convenient, however, and are generally recommended since they can monitor proteins at about 206–210 nm (to detect peptide bond carbonyl groups at maximum sensitivity), 220–225 nm (for peptide bond detection at reduced sensitivity), 280 nm (to detect proteins based on absorbance of tryptophan and tyrosine), or any other wavelength optimal for the solutes being detected. Some modern UV detectors also permit rapid spectral scans of peaks, monitoring of two wavelengths, or plotting of absorbance ratios. Detectors having photodiode arrays can monitor column effluent at multiple

wavelengths and subsequently process stored data with associated microcomputers.

DATA RECORDING AND PROCESSING

RP-HPLC data must be captured and somehow stored for evaluation. For many applications, a plot of absorbance vs time may suffice; the recorder scale is matched to the detector output but is frequently 10 mV full scale. Such data may be compared visually by superposition, and estimates of homogeneity and/or heterogeneity may be made; exact retention times and peak areas cannot be determined, however.

Inexpensive yet powerful integrators, which may also function as recorders, are frequently incorporated into HPLC systems. After each analysis, the integrator reports accurate retention times and peak areas or heights; peaks may also be identified and quantitated through built-in internal- or external-standard programs. After data are processed, however, they cannot be reintegrated if the initial parameters were not optimal.

Mainframe or personal computers are now used increasingly to store and process raw HPLC data. Recorders containing disk drives are also available, and system controllers may have data storage as well as processing functions. The decreasing costs of computers and of hard disk peripherals for storing large amounts of data make such functions more common. Major advantages are realized through the storage of raw data. If the initial integration parameters are not optimal, the data may be reintegrated. Also, various programs plot raw chromatographic data to any suitable scale, permitting chromatographic areas of interest to be expanded to scale and facilitating preparation of illustrations. Finally, when raw data are stored, chromatograms may be compared using suitable software; this may improve upon visual comparisons or may permit addition, subtraction, or ratioing of data. Such capabilities also permit baseline corrections, analysis of hybrids and mixtures, and statistical evaluation of data.

B. Columns for HPLC of Proteins

Numerous types of HPLC columns are now available for protein analysis (Wehr, 1984). Most columns consist of uniform porous silica microspheres, which can withstand high pressure. Thus, HPLC originally stood for "high pressure liquid chromatography." As particle uniformity increased, however, pressures also decreased, so today the "P" in HPLC stands for "performance." Silica supports are stable in most solvents in the pH range of about 2–8; nonsilica HPLC columns, useful over a wider pH range, are also available.

At least five types of silica-based HPLC columns exist, three of which are widely used for proteins. If columns (usually stainless steel) are packed directly with porous underivatized silica, adsorption columns result. Proteins may bind irreversibly to silanol groups of such columns, however, and such adsorption largely prevents the use of controlled-pore glass columns for cereal protein fractionation. A second type of wide-pore HPLC column derivatized to permit affinity chromatography of proteins has also been recently introduced.

In the three types of HPLC columns most useful for cereal proteins, most silanol groups are covalently modified to alter column functionality, and residual silanols are blocked by "end-capping" to minimize adsorption. If bonded phases

do not interact with proteins, the proteins separate by size, based on the relative penetration of the silica matrix, and SE-HPLC columns result. If silanols are derivatized with compounds having ionizable groups, IE-HPLC columns result. And if silicas are modified to incorporate hydrophobic, lipophilic groups, RP-HPLC columns result. "Reversed phase" is, however, really a misnomer. In classical adsorption chromatography on silica, "normal" chromatography involved columns having free silanols and nonpolar solvents; the "reverse" of this was a hydrophobic column, used with relatively polar (water-containing) solvents. "Hydrophobic interaction chromatography" (HIC) may thus be a more accurate term, but "HIC" now also designates elution by polar solvents (to minimize denaturation) from nonpolar columns. Thus, the term "reversed-phase" HPLC is now widely used and is used in this chapter.

Detailed consideration of all available protein-HPLC columns is beyond the scope of this chapter, and excellent recent reviews cover this topic (Hearn et al, 1982; Wehr, 1984). Nevertheless, important characteristics of such columns must be briefly described.

Most HPLC columns are made of silica because of its mechanical strength, ability to accurately control porosity and particle size, and ability to bond desired stationary phases to its surface. Most columns have uniform average particle diameters of ~5, 6.5, or 10 μm; pore diameters of 300 Å or more are most common, as they permit chromatography of large proteins. In contrast, most HPLC columns for separating LMW solutes have pores of 50-100 Å Covalent attachment, at high coverage, of organosilane stationary phases to silica silanol groups yields diverse columns differing in binding characteristics, for which ionic protein-silica interactions are minimized. All silanol groups are not derivatized, however, so "end-capping," in which a secondary silanization reagent such as trimethylchlorosilane blocks free silanols to prevent adsorption of proteins, is also used. Various "ion-pairing" reagents, such as trifluoroacetic acid (TFA), may also bind to free silanols, further reducing their reactivity toward proteins.

The type of stationary phase attached to silanol groups determines a column's character. Among reversed-phase columns, alkyl bonded phases such as C18, C8, C4, and C3 are generally most useful; cyanopropyl, diphenyl, and other column types may, however, differ in selectivity for proteins. Many wide-pore IE-HPLC columns are also available. Ion-exchange columns may be resin-based (as in traditional styrene divinylbenzene columns), polymer-based (such as some of Pharmacia's FPLC columns), or silica-based; each type varies in stability, application, suitability, and selectivity and may contain many weak or strong anion- or cation-exchange groups. SE-HPLC columns must have low ionic and hydrophobic character to minimize interactions, while providing a hydrophilic environment compatible with the mobile phase; frequently, bonded phases having hydroxylic moieties are used. A more detailed discussion of these factors is presented by Wehr (1984).

C. Techniques

SOLVENT PREPARATION AND COMPOSITION

Solvent purity is highly important for successful HPLC. Solvents must be sufficiently pure to have acceptable low absorbance at the wavelengths used for

monitoring samples and must have minimal amounts of dissolved solutes, which cause extraneous peaks. HPLC-grade solvents generally have such characteristics; spectrophotometric grade solvents may be unsuitable since they may have minimal absorbance but still contain interfering solutes. Preparing suitable solvents by distillation may be difficult, since interfering substances are frequently volatile organics.

Other components of HPLC solvents must also be highly pure. For example, solvent modifiers such as TFA should be of the highest available quality. This is important because TFA is the major contributor to solvent absorbance at ~210 nm, the wavelength commonly used to monitor eluting proteins. Although TFA (along with acetonitrile) (ACN) is most widely used in protein RP-HPLC, solvent modifiers such as heptafluorobutyric acid may modify column selectivity (for example, compare Figs. 4 and 5 in Bietz [1983]).

Water may be the most critical component of HPLC solvents. HPLC-grade water can be purchased, but it is expensive and may not always be suitable. It is preferable (and less expensive) to use a commercial water purification system having mixed-bed ion-exchange, organic removal (activated carbon), and filtration (e.g., 0.5-μm) cartridges. Water containing extremely low amounts of ionic, organic, and particulate components is thus available "on tap." If the water supplied to the apparatus is distilled water of reasonable quality, cartridges and filters last a long time, and such an apparatus requires little maintenance.

The final test of solvent suitability is the chromatogram itself: a blank chromatogram should be relatively "flat" and contain few if any peaks at normal detection levels. If contaminants are present in any solvent component, they may be concentrated under the initial column elution conditions and elute later during a gradient, giving the appearance of sample carry-over.

ACN is the most widely used organic phase in solvents for protein RP-HPLC. Blends of ACN and water are good solvents for most proteins, and ACN is sufficiently hydrophobic to elute most, if not all, polypeptides. Thus, gradients generated from water and ACN (or from mixed solvents such as 15% and 80% aqueous ACN) permit RP-HPLC analysis of most proteins, and these solvents are a reasonable initial choice for unknown samples. If desired separations cannot be achieved, solvents containing methanol, 2-propanol, or other organic phases may modify the selectivity.

HPLC solvents are prepared in two general ways. In the first, as used originally to study cereal proteins (Bietz, 1983), ACN, water, and TFA are combined to form limiting solvents (solvent "A" generally having lower hydrophobicity and "B" higher hydrophobicity) with hydrophobicities slightly lower (15% ACN) and higher (80% ACN) than needed to elute most proteins; such solvents also minimize bubble formation upon release of pressure in detector flow-cells. Bietz and Cobb (1985) later found, however, that reproducibility of elution times was not optimal using such solvents. Evaporation of the more volatile solvent components changes solvent hydrophobicity with time, delaying elution of proteins. Consequently, the solvents now recommended are water and ACN (containing equal amounts of an ion-pairing reagent, such as 0.1% TFA). Changes in solvent composition due to evaporation are then minor, and reproducibility of elution times is optimized (coefficients of variability are about 0.09). There is a slightly greater tendency for

bubbles to form in detector flow-cells with these solvents. This can be prevented by deaeration of solvents and by maintaining the flow-cell under slight (50–100 psi) pressure by a device such as about 1 m of capillary Teflon tubing attached via a union to the detector outlet.

Solvents are generally prepared the day they are used or on the previous day; some claim that solvents should "rest" for a day before use, but I see no advantage for this. Solvents must be prepared reproducibly. This can be achieved by using constant volumes of components (which is easy, of course, for simple solvents such as water + 0.1% TFA and ACN + 0.1% TFA). For greater accuracy in more complex blends, however, preparing solvents by weight rather than by volume may be advantageous. Solvents must be filtered before use, since particulates in solvents accumulate on filters, frits, guard columns, and/or columns, increasing the pressure and decreasing performance. Filtration under vacuum also deaerates solvents sufficiently for most purposes. Nylon filters (0.5-μm) are compatible with most HPLC solvents, and vacuum filtration through such filters removes most dissolved gases. Solvents may also be deaerated by other methods. Initially, Bietz (1983) used a high-powered ultrasonic probe immersed directly in the solvents. Although effective, this is generally unnecessary when flow-cells are slightly pressurized. Alternatively, solvents can be degassed by rapid bubbling for a few minutes with helium and can be maintained in this condition by slow sparging with helium under a helium atmosphere. When solvents are used while reasonably fresh, however, this procedure (which increases the complexity and cost of the system) can often be eliminated. Ideally, solvents are made fresh daily, but they can generally be used for at least three days, representing unattended operation during a weekend. To compare samples directly, the same solvent batch should be used to minimize differences in retention caused by slight differences in solvent composition.

SAMPLE PREPARATION AND APPLICATION

Nearly any solvent suitable for HPLC analysis, including the chromatographic solvents themselves, may be used for cereal protein extraction or solubilization. Solvents should generally have a pH of 2–8, in which silica is stable; injection of small volumes of slightly more acidic or basic samples may be tolerated, however, since sample solvents are rapidly eluted upon injection.

A few examples of extraction conditions for cereal protein HPLC will show more specific details.

Bietz et al (1984a) determined optimal conditions for extraction of wheat gliadin for HPLC varietal identification. Effects of extraction time, defatting, prior NaCl extraction, type of extractant, and age of extract were systematically explored. Optimal conditions were found to be similar to the simple conditions used to extract gliadin for other analyses. Defatting and NaCl extraction are generally unnecessary. A 30-minute extraction is sufficient (although more rapid extraction can be used, or yields can be increased slightly using longer extraction times). Aqueous 70% ethanol and 55% 2-propanol are good extractants. Samples are stable for at least one month. (Some association or precipitation may occur if samples are frozen; thus, storage at room temperature is generally recommended, providing that samples are sufficiently stable under such conditions. Most prolamin and glutelin subunit samples are quite stable; albumin and globulin extracts are not, however, because proteases may be

present or disulfide cross-linking through thiol groups may occur.) Solvents such as aluminum lactate buffer (p H 3.1), 2 M urea, and 2 M dimethylformamide are less acceptable than aqueous alcohols because nongliadin proteins, including native HMW glutenins, which may poorly resolve, are also extracted, diminishing the apparent resolution of the resulting chromatograms. This extraction procedure can be used with either flour or crushed single kernels. Alternatively, isolated proteins may be redissolved and then analyzed after centrifugation or filtration (Bietz, 1983).

Lookhart (1985a) and Lookhart and Pomeranz (1985a) also used RP-HPLC for varietal identification of oat cultivars and species. For this purpose, avenins were extracted for one hour with aqueous 70% ethanol.

Corn prolamins and alcohol-soluble glutelin (ASG) subunits may also be simply extracted for RP-HPLC analysis. Bietz (1985b) showed that zein may be reproducibly extracted from ground single kernels with 70% ethanol. Paulis and Bietz (1986) demonstrated that the total alcohol-soluble protein fraction (zein plus alcohol-soluble reduced glutelin subunits) can be extracted with 70% (v/v) aqueous ethanol–0.5% NaOAc–5% (v/v) 2-mercaptoethanol (ME) for two hours. In these and other cases, samples may be prepared for injection by centrifugation (typically 10 minutes at 36,000 × g) or by filtration (0.45 μm).

Prolamins, ASG subunits, or the combined alcohol-soluble polypeptides of other cereals may also be easily extracted for varietal identification. Marchylo and Kruger (1984) extracted barley proteins with 50% 1-propanol containing dithiothreitol, ME, or monothioglycerol as reducing agents; they also extracted wheat proteins with 50% 1-propanol containing 1% acetic acid plus dithiothreitol (Kruger and Marchylo, 1985). These extracts, containing monomeric prolamins plus ASG subunits, were useful for RP-HPLC varietal identification. Sorghum kafirins may be extracted with aqueous 60% *tert*-butanol (Sastry et al, 1986a), and alcohol-soluble sorghum glutelin subunits may subsequently be extracted (for two hours) with 60% *tert*-butanol + 2% ME; alternatively, kafirins plus ASG subunits may be coextracted with 60% *tert*-butanol + 2% ME (Sastry et al, 1986b). Alkylation of reduced sorghum proteins with acrylonitrile improved the resulting RP-HPLC separations (Sastry et al, 1986b), probably by stabilizing thiol groups.

Other cereal proteins may also be easily extracted and analyzed by RP-HPLC. Albumins or globulins may be extracted with water or dilute saline solutions (Bietz, 1983). Similarly, Burnouf and Bietz (1984b) determined the optimal conditions for extracting wheat glutenin for RP-HPLC analysis. Glutenin was first isolated from bulk samples, using various dissociating, reducing, and alkylating conditions; subunits were subsequently separated by RP-HPLC on several columns. Excellent separations occurred when glutenin was dissociated with 8 M urea or 6 M guanidine hydrochloride, reduced with 5% ME or 0.1% dithiothreitol, alkylated with 4-vinylpyridine, and chromatographed on C18 or C3 columns. Samples were stable for one month at room temperature, even in the presence of urea and components of the alkylation reaction mixture. Although alkylation is necessary for optimal resolution of glutenin subunits, researchers are not certain whether it improves separations through cysteine modification or prevents reassociation of subunits through disulfides during RP-HPLC under nonreducing conditions. These conditions have been used to isolate glutenin subunits from single wheat kernels for RP-HPLC aneuploid

analysis (Burnouf and Bietz, 1985).

Simple techniques can also extract and solubilize cereal proteins for SE-HPLC or IE-HPLC. Batey (1984) extracted wheat gliadins with 70% ethanol (following preliminary salt extraction) and, after drying them, resuspended them in the initial IE-HPLC solvent ($1 M$ urea plus basic buffer), filtered them, and applied them to the column. For SE-HPLC (Bietz, 1984a), samples were dissolved in chromatographic solvent to prevent baseline anomalies. One suitable SE-HPLC solvent is 0.1% sodium phosphate, pH 7.0, containing 0.1% SDS plus sufficient added SDS so that at least twice as much SDS is present as protein; 1% ME may also be added to reduce disulfide bonds, thus dissociating proteins to their subunits. Alternatively, flour or ground meal samples may be directly extracted with this solvent. When solvents contain SDS, it is essential that the proteins fully complex to SDS so that molecular weight may be related to elution volume; this can be achieved by incubation at room temperature overnight, at 50–60° C for one to two hours, or in a boiling water bath for about five minutes; lower temperatures must be used with flour and meal samples to avoid starch gelatinization. For preparation of some samples for SE-HPLC, inclusion of urea during complexing to SDS may also be desirable, as for SDS-PAGE.

Introduction of samples into an HPLC system is extremely simple using manual or automatic injectors. It is very important for samples, as for solvents, that all particulate matter be removed before analysis. This can be achieved by centrifugation (10–20 minutes at 25,000–400,000 $\times$ g) or by filtration; centrifugation is most convenient and inexpensive for numerous samples, but filtration (for instance, with disposable syringe-type filters) may be preferable for rapid analyses.

After being introduced into an HPLC system, a sample almost immediately equilibrates with chromatographic solvents. Although proteins are retarded upon injection, buffers, salts, and ionic sample and solvent components generally do not bind but elute at the solvent front. Because of this, samples may generally be dissolved in any appropriate solvent between pH 2 and 8. The sample volume may also vary greatly. In SE-HPLC, minimal sample volume is used to minimize band spreading. In RP- or IE-HPLC, however, where stacking of proteins generally occurs upon injection, sample volumes from microliters to milliliters can be used. Application of large volumes of sample may also eliminate sample concentration steps for preparative and semipreparative applications.

The amounts of protein analyzed by HPLC can also vary widely. For analytical separations, Bietz (1983) first used 0.1 mg of heterogeneous samples, dissolved in 100 μl, with a detector absorbance range of 0.2 absorbance units full scale (AUFS); the sample amount could, however, be increased or decreased by one to two orders of magnitude. Thus, 5 μg of an isolated protein may easily be analyzed, and this amount may be reduced to 50 ng by increasing the detector sensitivity. Proteins extracted from single cereal grains may easily be analyzed; indeed, sequential extraction may be performed on single kernels and each fraction characterized by HPLC. Alternatively, 5 mg can be analyzed on analytical columns and 25–50 mg on semipreparative ($\sim$10 mm i.d.) columns.

As noted above, UV absorbance detectors generally monitor proteins in HPLC; thus, the operator need only choose the wavelength and absorbance

range to be used. For maximum sensitivity and for quantitation, wavelengths of 206–210 nm are most common; in this range, peptide-bond carbonyl groups absorb (as do some amino acid sidechains). If the sample size is large and precludes the use of 206–210 nm even at low sensitivity (e.g., 2.0 AUFS), the effluent may be quantitatively monitored at 220–225 nm at reduced sensitivity. For preparative separations, or when detection of tryptophan and/or tyrosine is desired, the effluent may be monitored at 254 or 280 nm. It may also be desirable, using modern detectors, to monitor two or more wavelengths simultaneously. Such a capability could permit the monitoring of proteins at different sensitivity settings, detection of only aromatic amino acids, or plotting of absorbance ratios at any two wavelengths, which may be useful to differentiate proteins having different compositions.

Absorbance ranges of 0.1–0.4 AUFS, on a 10-mV recorder, are most common for cereal protein HPLC. This range can be varied widely, but, at high detector sensitivity, noise increases and the ratio of signal to noise decreases.

TEMPERATURE EFFECTS IN HPLC

Temperature has two major effects in cereal protein HPLC. First, constant temperatures must be maintained to maximize reproducibility. Since varying the temperature between 20 and 40° C had little effect on separations, 30° C was originally used for RP-HPLC (Bietz, 1983). At times, columns also operated at ambient temperature. When RP-HPLC reproducibility was examined in greater detail, however, temperature was found to have a marked effect (Bietz and Cobb, 1985): daily cyclic temperature fluctuations in a laboratory alter elution times, decreasing reproducibility. Typically, coefficients of variability at constant temperature are about 0.1, but they vary from 0.3 to 0.9 at ambient temperature (Bietz and Cobb, 1985).

Resolution of many cereal proteins also markedly improves at increased temperature (Bietz, 1984c, 1986; Bietz and Cobb, 1985). As shown in Figure 1, the resolution for gliadins constantly improved from 20 to 70° C; resolution at 60 and 70° C was much better than at 50° C, so 70° C is now generally used for RP-HPLC of cereal proteins. This improved resolution at elevated temperature may indicate that cereal proteins interact through hydrogen bonds in associated or aggregated samples: interactions due to hydrogen bonding decrease in strength as temperature increases (Cole et al, 1983), whereas hydrophobic interactions become stronger (Tanford, 1970).

USE OF STANDARDS IN HPLC

As in any analytical procedure, standards must periodically be analyzed to calibrate columns, to monitor column and system performance, and to compare results obtained under different conditions. In SE-HPLC (Bietz, 1984a), standards are generally proteins of known molecular weight: logarithms of molecular weight, plotted against elution times, give a linear relationship from which the molecular weight of unknown proteins can be accurately predicted. At the same time, consistent elution times for known proteins indicate that the system is performing properly.

RP-HPLC standards may be of two types. Perhaps the more useful is a heterogeneous sample similar to those usually analyzed. Changes in elution time indicate problems with pumps (as when a bubble is trapped in the heads) or

leaks, resulting from bad fittings, whereas changes in resolution may indicate contamination or aging of columns. Such a standard should be freshly prepared at the same time as the samples, from a bulk sample known to be stable (e.g., a frozen flour), using identical extraction and sample preparation conditions. These precautions will ensure that any observed differences are due to the column or system and not to the sample.

Another type of standard that may prove useful in RP-HPLC is a mixture of LMW solutes such as alkylphenones (Kikta and Stange, 1977; Bietz and Cobb, 1985) that vary in hydrophobicity because of differences in alkyl chain length. Analysis of such compounds added to samples (internal standards) or analyzed in an identical run (external standards) could compensate for different elution characteristics of proteins on columns of different types or columns of the same type that differ in age or resolving power. The elution positions of sample components can then be expressed in terms of standards, as by a "hydrophobicity index" (Bietz and Cobb, 1985), rather than in terms of time or of ACN concentration at time of elution. Elution characteristics of alkylphenones and of gliadins may vary somewhat independently upon
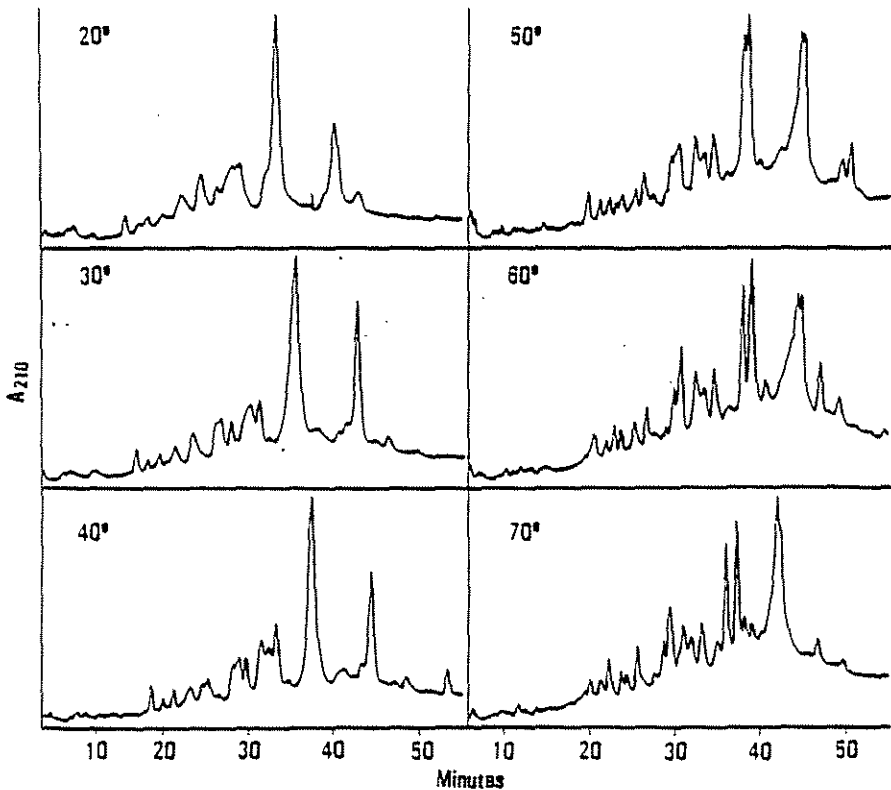

Figure 1. Effect of temperature on reversed-phase high-performance liquid chromatographic resolution of Centurk wheat gliadin. Aliquots of a fresh 70% ethanol extract were applied to an Altex Ultrapore-RPSC (C3) column at temperatures from 20 to 70° C and eluted with 25-45% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes. (Reprinted, with permission, from Bietz, 1986)

RP-HPLC, however, which complicates the use of alkylphenones as standards (J. R. S. Ellis, personal communication).

USE OF COMPUTERS IN HPLC

For many applications, visual inspection of chromatograms can characterize, compare, or identify samples. Usually, however, further data processing is necessary or desirable. This can be most simply achieved using an electronic integrator, which provides accurate elution times and peak areas; sample identities, component amounts, or other information may also be reported.

Frequently, however, computers, including relatively small personal computers, are now used to store, process, evaluate, and compare chromatographic data. The use of computers in HPLC has several advantages.

Reintegration. Chromatographic integrators analyze data after each run, converting it to stored elution times and areas. To best analyze the data, all integration parameters must be optimal, and if optimal integration is not achieved, reanalyzing the sample may be necessary. If, however, "raw" data points, consisting of periodic detector voltage readings, are stored, data may be reintegrated using optimal parameters. "Hard disks" in a computer store large amounts of data in this manner. For archival storage, data can be transferred to magnetic tape or floppy disks, from which it can be later restored to the computer.

Replotting. Examination and comparison of HPLC data on a recorder may be difficult because elution times of peaks may be close, peak heights may vary due to different sample amounts, and peaks may go off scale. These problems can be largely prevented when raw chromatographic data are stored. Software is available, for both mainframe and personal computers, that permits replotting of any portions of chromatographic data to any convenient scale. It is thus possible, for example, to eliminate the solvent peak at the void volume and to magnify small peaks for more careful examination (Bietz, 1983; Marchylo and Kruger, 1985). The voltage range of the computer may be greater than that of the recorder, so "off scale" data is not lost. Equivalent peaks in chromatograms may also be plotted to the same heights. Storage of raw data in this manner also facilitates preparation of illustrations.

Comparison of Chromatograms. One of the most powerful capabilities of computer analysis of raw data involves comparison of different runs. For this purpose, we use the program "CHROCP" (CHROmatographic Comparison Plot), developed by R. Butterfield at the Northern Regional Research Center, which permits addition and subtraction of chromatograms in several ways. In the "unity" mode, voltage readings of one data set are subtracted directly from those of another chromatogram; this is most useful for correcting sloping baselines or eliminating extraneous peaks from blank runs. CHROCP operates by setting the value of K in the equation

$$A - KB = C,$$

where A = chromatogram 1, B = chromatogram 2, and C = the computer-generated "difference" chromatogram resulting from applying this equation at each chromatogram time interval. Thus, in the unity mode, the computer sets K to equal 1. In the "automatic" mode, the value of K is determined by the computer so that the negative deflections of C from its baseline are minimized.

This may be useful for analyzing mixtures: subtraction of component *B* from mixture *A* gives a chromatogram *C* representative of the other component of a binary mixture, and the value of K indicates the relative amounts of each component. In the "manual" mode of CHROCP, K is set to any desired positive or negative value; this permits normalization of total chromatographic areas, for example, as well as addition of chromatograms when K is negative. This portion of the program is useful for hybrid analysis, since total chromatographic areas of inbreds may be adjusted to the 2:1 contribution of maternal to paternal genes for endosperm proteins, and since computer-generated patterns may be compared directly to chromatograms of proteins from authentic hybrids to study the regulation of protein expression and hybrid pedigree. Finally, in the "cursor" mode of CHROCP, the difference between *A* and *B* at any time is set to zero. This option is useful in comparing aneuploids or closely related genotypes, since when the difference is zero at points known (or thought) to be identical, components exhibiting the greatest differences are revealed by positive or negative deflections from the baseline.

In addition to these capabilities, computers may be valuable in searching for relationships among the complex data for numerous samples. We have, for example, found (as detailed below) that various RP-HPLC peaks are related to cereal quality; the ability to statistically evaluate stored data by computer should reveal additional quality relationships by optimizing correlations between peak elution times, areas, or heights and independently measured quality characteristics. Alternatively, by determining correlation coefficients between detector response and measured characteristics at each time interval, one may be able to detect quality-related components, which could then be isolated for further characterization; further rapid HPLC quality tests could then be devised. Close linkage has been found among genes that code many cereal storage polypeptides (Sozinov and Poperelya, 1980; Branlard, 1983; Payne et al, 1984), mainly through coinheritance of proteins as demonstrated by electrophoresis; similar relationships should be detectable by HPLC.

Several attempts have been made to automate cereal variety identification through computer analysis of electrophoresis data (Wrigley, 1980; Lookhart et al, 1983; Sapirstein and Bushuk, 1985); in most cases, however, some subjective interpretation of band intensity is necessary. HPLC, with the aid of computers, may eliminate such subjective evaluation and permit automated varietal identification through comparison of chromatograms to a library of standards. This should be possible since reproducibility of HPLC is often better than that of electrophoresis, and since quantitation at ~210 nm is better than that of methods using various protein stains, which may bind to only certain amino acids and give different responses for different proteins.

D. Advantages of HPLC

HPLC has distinct advantages for fractionation of cereal proteins, as summarized in this section.

SPEED
Compared to most methods, HPLC is very fast. Optimal RP-HPLC resolution generally requires one to two hours; at higher temperatures, 10–15-

minute separations are often adequate. For some purposes, such as rapid screening of durum wheat varieties for pasta quality (Burnouf and Bietz, 1984a), gradients of about five minutes may even reveal desired sample characteristics. Similarly, run times for SE-HPLC are generally only 20–30 minutes (Bietz, 1984a), and for IE-HPLC, run times of 15–20 minutes have been reported (Batey, 1984). In contrast, run times for comparable separations by gel filtration, ion-exchange chromatography (IEC), or hydrophobic interaction chromatography (HIC) frequently take days, rather than hours or minutes. Rapid analyses are primarily due to small, uniform silica-based column packings, which permit high linear flow rates without compressing supports.

SENSITIVITY
Because of small, efficient columns and the ability (with many solvents) to monitor proteins at ~210 nm, cereal protein HPLC requires very small samples. For RP-HPLC, for example, as little as 50 ng of protein per peak may be detected, and 5-μg quantities (correspondingly more for complex mixtures) are easily analyzed. This high sensitivity permits, for example, numerous analyses of proteins to be made from a half kernel of grain, while the germ may be saved for further evaluation.

RESOLUTION
Resolution of cereal proteins by HPLC generally equals and often far exceeds that of conventional chromatography. In RP-HPLC, for example (Bietz, 1983, 1984c, 1985a, 1986), 40–100 peaks commonly resolve from complex mixtures in about one hour; this far exceeds resolution by HPLC's conventional counterpart, HIC, on porous agarose columns (Popineau and Godon, 1978; Caldwell, 1979; Chung and Pomeranz, 1979). In addition, the resolution of RP-HPLC generally exceeds that of one-dimensional electrophoresis and approaches that of many two-dimensional electrophoresis methods. Resolution of SE-HPLC (Bietz, 1984a), however, is similar to that of conventional gel filtration; the major advantage of SE-HPLC is speed. For IE-HPLC (Batey, 1984), initial results suggest that resolution is similar to that on conventional cellulose IEC columns (e.g., Huebner and Wall, 1966; Charbonnier, 1974). IE-HPLC seems to have considerable potential for further improvement.

REPRODUCIBILITY
Excellent reproducibility can be achieved by HPLC, primarily because the solvent delivery systems and controllers permit accurate and constant flow rates and gradients. Two types of modifications can further improve reproducibility (Bietz and Cobb, 1985). First, constant column temperature is essential to maximize RP-HPLC reproducibility.

Similarly, Bietz and Cobb (1985) noted, using a gradient generated from the solvents 15% ACN + 0.1% TFA and 80% ACN + 0.1% TFA, that retention times increased in replicate RP-HPLC chromatograms due to preferential evaporation of ACN from solvents. A similar problem was noted by Marchylo and Kruger (1984) and was alleviated by controlling solvent evaporation. Bietz and Cobb (1985) found, however, that changing solvents to water + 0.1% TFA and ACN + 0.1% TFA also eliminated the problem and that it reduced the coefficient of variability for elution times to ~0.09. With these solvents,

evaporation does not significantly alter solvent composition or retention times. The slightly greater tendency toward outgassing of these solvents in detector flow-cells is readily controlled by maintaining the flow-cell under slight pressure and by deaeration of solvents.

RECOVERY

Few studies have yet considered HPLC recovery in detail, but it generally seems excellent. Bietz (1983) found, by comparing absorbance of applied and recovered samples, that 96–98% of applied proteins were recovered in RP-HPLC; other studies of noncereal proteins report recoveries of 80–100% for proteins and large peptides from porous RP-HPLC columns, suggesting that good recovery is a general phenomenon when protein adsorption to silanols is prevented by end-capping.

QUANTITATION

One of the greatest advantages of HPLC is that data are easily quantitated, generally more accurately than in standard chromatography or electrophoresis. Constant flow rates, rapid analyses, and reliable injection and integration systems partially explain this capability; more important, however, is the monitoring of carbonyl groups of peptide bonds in column effluents at ~210 nm. Consequently, all protein amino acids are detected (plus some side chains), rather than only certain amino acids (primarily tryptophan and tyrosine) at 254 or 280 nm. Integration of HPLC data is also generally superior to that of electrophoresis data, where nonlinearity and limited ranges of staining and densitometry methods, plus adsorption of protein stains to only certain (generally basic) amino acids, combine to make accurate quantitation of electrophoresis data difficult, questionable, or impossible.

AUTOMATION

The availability of system controllers and automatic sample injectors in modern HPLC systems greatly increases the number of samples that may be analyzed by night or weekend operation, while reducing the possibility of operator error and releasing time for other duties. In addition, reproducibility improves because of constant reequilibration conditions for each sample, and automation ensures accurate and reproducible sample injection volumes.

EASE, SIMPLICITY, AND RELIABILITY

Operation of most HPLC systems is relatively easy and can be taught (or learned) in a few hours. HPLC also seems to require less "art" than does electrophoresis, which may permit development of standard HPLC methods.

PREPARATIVE CAPABILITIES

Bietz (1983) found that up to 5 mg of gliadin or zein could be fractionated on analytical (~4 mm i.d.) RP-HPLC columns while adequate resolution was maintained; on semipreparative (~10 mm i.d.) columns, samples of 25–50 mg may be fractionated (Burnouf and Bietz, 1984a; Huebner and Bietz, 1984; Paulis and Bietz, 1985). Obviously, larger quantities could be separated on still larger columns, keeping the ratio of sample to cross-sectional area approximately constant and generally also keeping the linear flow rate constant. The rapid

analysis times (typically 30–120 minutes) of HPLC, combined with automated sample injectors and "intelligent" fraction collectors (capable of detecting, collecting, and combining desired fractions from numerous runs), permit unattended preparative HPLC, even using analytical columns.

COLUMN STABILITY

The covalent attachment of stable bonded phases to physically strong support particles, combined with the use of guard columns and in-line filters to protect the column from particulates and solutes that adsorb irreversibly to packings, generally lead to excellent column stability and lifetime. Hundreds or even thousands of analyses may then be achieved before performance noticeably deteriorates or resolution is lost. Thus, even though most (~4 × 250 mm) reversed-phase or ion-exchange columns cost $200–400, the cost per analysis, especially when compared to that of other chromatographic supports, is low. SE-HPLC columns are generally larger and more expensive ($600–1,000) than other HPLC columns, but the savings in time (15–30 minutes per analysis vs several hours or even days using conventional gel filtration columns) easily justifies their use.

COMPLEMENTARY NATURE OF RP-HPLC

Although IE-HPLC and SE-HPLC separations generally duplicate (except for above mentioned advantages) those of conventional IEC or gel-filtration chromatography, respectively, RP-HPLC considerably surpasses its counterpart, HIC, in resolution. Thus, one of the most significant advantages of RP-HPLC over other procedures is that it complements methods based on size or charge differences, permitting separations based on surface hydrophobicity unobtainable by any other method.

LIMITATIONS

Of course, HPLC also has some limitations: equipment is mechanically and electronically complex and consequently quite expensive (although costs are decreasing and the equipment is generally highly reliable). Also, procedures permitting interlaboratory comparisons of data through use of internal standards are not yet totally proven—HPLC of cereal proteins is a recent and still evolving technique. Obviously, HPLC cannot, and is not meant to, replace any other method; its very complementary nature makes it a valuable addition to existing methods. For some applications, however (see below), HPLC methods may be advantageous to those currently used.

E. Standard Conditions for Protein HPLC

COLUMN TYPES AND SIZES

As noted above, most RP- and IE-HPLC columns have an internal diameter of ~4–4.6 mm and are 250 mm in length, but shorter (e.g., 75 mm) analytical columns are also available, as are semipreparative (e.g., 10 × 250 mm) and larger preparative columns (Wehr, 1984). SE-HPLC columns are larger (e.g., 7.5 mm i.d. × 500–600 mm), however, due to the greater dependence of resolution on column size. In RP-HPLC, C18, C8, C4, and C3 columns are the most widely used; silicas generally have pores of ~300 Å, and particle sizes are 5, 6.5, or 10

μm (Wehr, 1984). Other column types, although less widely used, may facilitate difficult separations because of their different selectivities.

Another type of HPLC column for proteins is also now available. This hydrophobic interaction column is similar to an RP-HPLC column in mode of separation, i.e., surface hydrophobicity, but buffered solvents containing no organic phase are generally used, and solutes are eluted by a gradient of decreasing salt concentration (Goheen and Stevens, 1985). Such columns may preserve the activity of enzymes but have not yet been tested with cereal storage proteins. Good separations may result, but the relatively high organic content of most RP-HPLC solvents generally favors cereal protein solubility, and most cereal storage proteins have limited solubility in solvents containing high concentrations of salt. In addition, enzymatic activity and/or denaturation of cereal proteins is usually of little or no concern.

SOLVENT AND GRADIENT CONDITIONS

ACN, used with TFA and water, is probably the most widely used organic phase in RP-HPLC. It is an excellent solvent for most polypeptides and is sufficiently hydrophobic to elute most, if not all, proteins of any type from RP-HPLC columns. ACN also exhibits relatively low viscosity compared to that of solvents such as 2-propanol, especially at elevated temperatures. TFA is most widely used as an RP-HPLC ion-pairing or selectivity modifying agent, generally at a concentration of 0.1% (Bietz, 1983); 0.05% TFA is also sufficient (Huebner and Bietz, 1984). Higher TFA concentrations (0.2–0.3%) have also been used (Bietz, 1983), leading to slight differences in selectivity. Lookhart has reported (personal communication) that solvents containing high concentrations of TFA (0.2–0.5%) may improve resolution; high TFA concentrations (>0.5%) may, however, not always permit monitoring of the effluent at ~210 nm, since TFA is the main contributor to the 210-nm absorbance of blends of ACN, TFA, and water. Some concern that protein deamidation may occur at high TFA concentrations has also been expressed (Huebner and Bietz, 1984).

In addition to ACN-TFA-water solvents, other organic phases and ion-pairing reagents may be used. For example, Bietz (1983) found that methanol or 2-propanol could be substituted for ACN, heptafluorobutyric acid (HFBA) could replace TFA, and detergents such as SDS could be used in RP-HPLC solvents. Some differences in selectivity occurred, suggesting that solvent modification may facilitate difficult separations.

Solvents for IE-HPLC and SE-HPLC are generally similar to those used with nonsilica columns. For example, Bietz (1984a) used primarily neutral $0.1M$ sodium phosphate buffer, with or without SDS (generally 0.1%) and reducing agent (ME or dithiothreitol), for SE-HPLC. Batey (1984) used several alkaline buffers, in the presence of $1M$ urea and a linear gradient of $0–0.5M$ sodium acetate, for IE-HPLC of wheat gliadins. In short, essentially any solvents in the pH stability region of silica (2–8) may be used with silica-based HPLC columns.

FLOW RATES AND GRADIENT CONDITIONS

For most analytical (4.0–4.6 × 250 mm) RP-HPLC, IE-HPLC, and SE-HPLC separations, flow rates of ~1.0 ml/min are used; such columns have holdup (void) volumes of 2–3 ml, and, for separations of 30–60 minutes,

resolution does not improve significantly at higher or lower flow rates. SE-HPLC columns frequently have void volumes of 6–8 ml and total volumes of 20–25 ml, permitting 20–30-minute separations at 1.0 ml/min. Higher SE-HPLC resolution of proteins has been claimed at lower flow rates, but such improvements have not been observed for cereal proteins (Bietz, 1984a). For semipreparative (e.g., 10 mm i.d.) RP-HPLC columns, flow rates are generally 2.5–3.0 ml/min, to provide linear flow rates approaching those of analytical columns and to more rapidly elute solutes displaced by the gradient.

With ACN RP-HPLC solvents, all cereal proteins have been found to elute between 15 and 80% ACN. Thus, these ACN concentrations were originally chosen as limiting in solvents for cereal protein RP-HPLC, since they could be used to form any suitable gradient (Bietz, 1983). More recently, however, water + 0.1% TFA was used as solvent A and ACN + 0.1% TFA as solvent B to significantly improve reproducibility (Bietz and Cobb, 1985). Of cereal polypeptides, albumins and globulins generally have the lowest surface hydrophobicities; many thus elute first upon RP-HPLC, but others elute over a fairly wide hydrophobicity range (Bietz, 1983). Certain nonprolaminlike subunits of glutelins generally elute next: these include HMW wheat glutenin subunits (Burnouf and Bietz, 1984b, 1985), as well as high-methionine and high-histidine/proline subunits of maize glutelin (Paulis and Bietz, 1986). Finally, true prolamins generally have the highest surface hydrophobicities among cereal proteins and elute last among proteins from any cereal. For example, wheat gliadins elute at a maximum ACN concentration of 50–60%, but maize zeins require 60–70% ACN for displacement from C18 columns. For wheat polypeptides, Bietz and Burnouf (1985) suggested, based on the observation that most protein classes elute in distinct hydrophobicity ranges, that polypeptide type can often be predicted from RP-HPLC elution characteristics.

TEMPERATURE EFFECTS

As noted above, constant temperature maximizes RP-HPLC reproducibility, and elevated temperature (60–70° C) often significantly improves cereal protein resolution (Figure 1). For gliadins and other cereal proteins tested, the resolution also becomes progressively worse as temperatures decrease (Bietz, 1986; Bietz and Cobb, 1985).

Jones et al (1985), however, noted that RP-HPLC resolution of wheat α-purothionins is significantly worse at 40° C than at 20° C and that chromatography at 0° C results in baseline separations. This was tentatively attributed to the stabilization of purothionin conformations at lower temperatures, resulting in narrow peaks. Since hydrophobic bonding weakens at lower temperatures, dissociation of hydrophobically associated purothionins may be facilitated under these conditions; this seems unlikely, however, considering their amino acid compositions. Thionins differ significantly from most cereal storage proteins, having relatively low molecular weights and high contents of basic amino acids and different structures and properties. These results emphasize, then, the importance of examining temperature effects in any new RP-HPLC separation of cereal proteins; improved resolution may well result through temperature optimization.

ANALYSIS TIME

In initial RP-HPLC studies of cereal proteins (Bietz, 1983), analysis times of 60–120 minutes generally provided optimal resolution; 30-minute separations were also possible. Increased RP-HPLC resolution at elevated temperatures (Bietz and Cobb, 1985) now makes rapid separations more useful and achievable. For example, 55- and 20-minute separations of wheat gliadins were nearly comparable, and even 10-minute separations could (although resolution did decrease) differentiate U.S. wheat cultivar biotypes and identify English wheat varieties (Bietz and Cobb, 1985). Similarly, Burnouf and Bietz (1984a) found that five-minute separations could differentiate durum wheat quality types.

An example of the effect of analysis time on cereal protein resolution is shown in Figure 2. Zeins from maize inbred W64A were analyzed under conditions identical except for gradient time, which ranged from 60 to 10 minutes. Although resolution steadily decreases with gradient time, most major peaks in the 60-minute run are still apparent using 10–20-minute gradients. This suggests that RP-HPLC, as well as other rapid HPLC methods—e.g., 20–30 minutes for SE-HPLC (Bietz, 1984a) or IE-HPLC (Batey, 1984)—can be used for routine quality control, selection, and identification.

OPTIMIZATION OF ANALYSIS CONDITIONS

A simple two-step procedure can generally suggest nearly optimal RP-HPLC conditions for any new sample type. As noted above, ACN-TFA-water solvents should be used first; solvent A should preferably be water + 0.1% TFA, and solvent B, ACN + 0.1% TFA. Initial conditions could be 0–100% B during 60 minutes at 1.0 ml/min and 70°; detector wavelength should be 210 nm, using 0.2–0.4 AUFS. Initial results can then be used to predict the appropriate ACN gradient for subsequent runs. Generally, gradients of 0.25–0.5% ACN per minute give maximum resolution; this may be increased to 2–3% ACN per minute (at 2.0–3.0 ml/min) to maximize speed while retaining sufficient resolution for many purposes. A final hold of 10 minutes at maximum ACN concentration generally ensures elution of the most hydrophobic proteins, and reequilibration for 10 minutes at the initial gradient conditions prepares the column for the next analysis. If further improvement in separation is needed, one should vary the run time, test different organic phases (e.g., 2-propanol) or ion-pairing reagents (e.g., HFBA), or use columns with different selectivities.

Bietz et al (1983) first noted that RP-HPLC selectivity for cereal proteins was modified significantly on columns other than C18. By examining a standard set of heterogeneous cereal protein samples on porous C18, C8, C4, C3, cyanopropyl, diphenyl, and phenyl RP-HPLC columns from several sources, they found that resolution and selectivity varied significantly. Alkyl (C18, C8, C4, and C3) columns generally gave best resolution, and columns of comparable bonded phase from different manufacturers were similar. Replicate columns from the same source gave nearly identical separations.

Typical separations of the same sample (pyridylethylated glutenin subunits) on different RP-HPLC columns are shown in Figure 3. Only minor differences occur among alkyl columns (see arrows a–c), but some may be significant. Retention times and resolution on other column types vary considerably, however. These chromatograms were run under identical conditions, which may
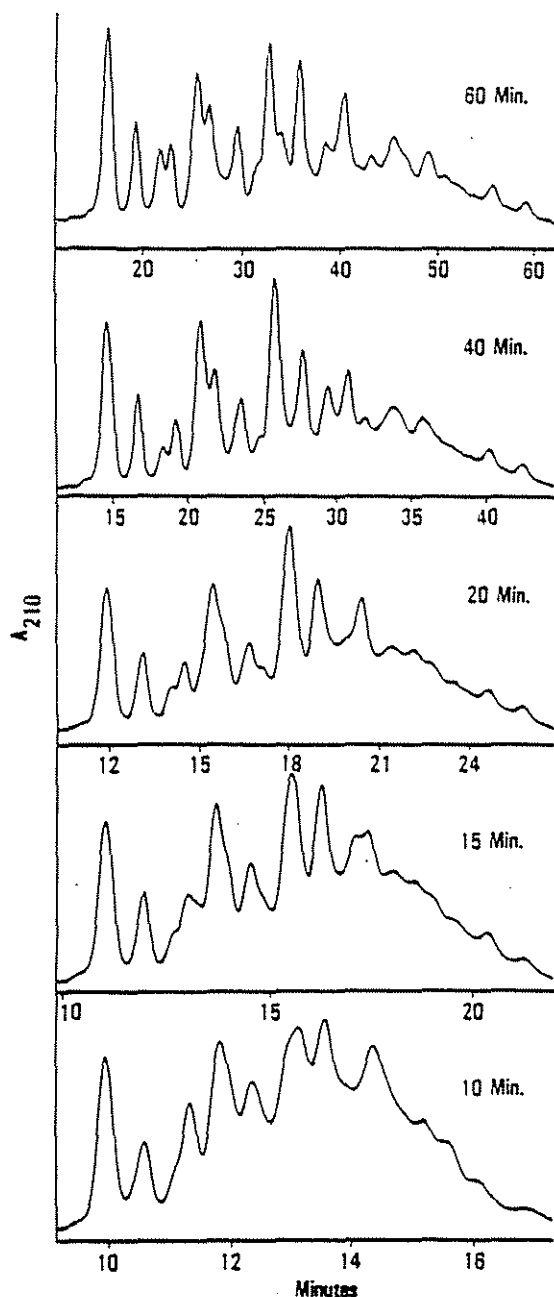
Figure.2. Effect of gradient time on reversed-phase high-performance liquid chromatographic resolution of zeins from maize inbred W64A. Zein was extracted with 70% ethanol (1.0 ml per ground kernel), centrifuged, applied (50 μl) to a SynChropak RP-P (C18) column at 70°C, and eluted at 1.0 ml/min with 48–58% acetonitrile (+0.1% trifluoroacetic acid) during 60, 40, 20, 15, and 10 minutes, with final gradient holds at 58% acetonitrile to elute all components.
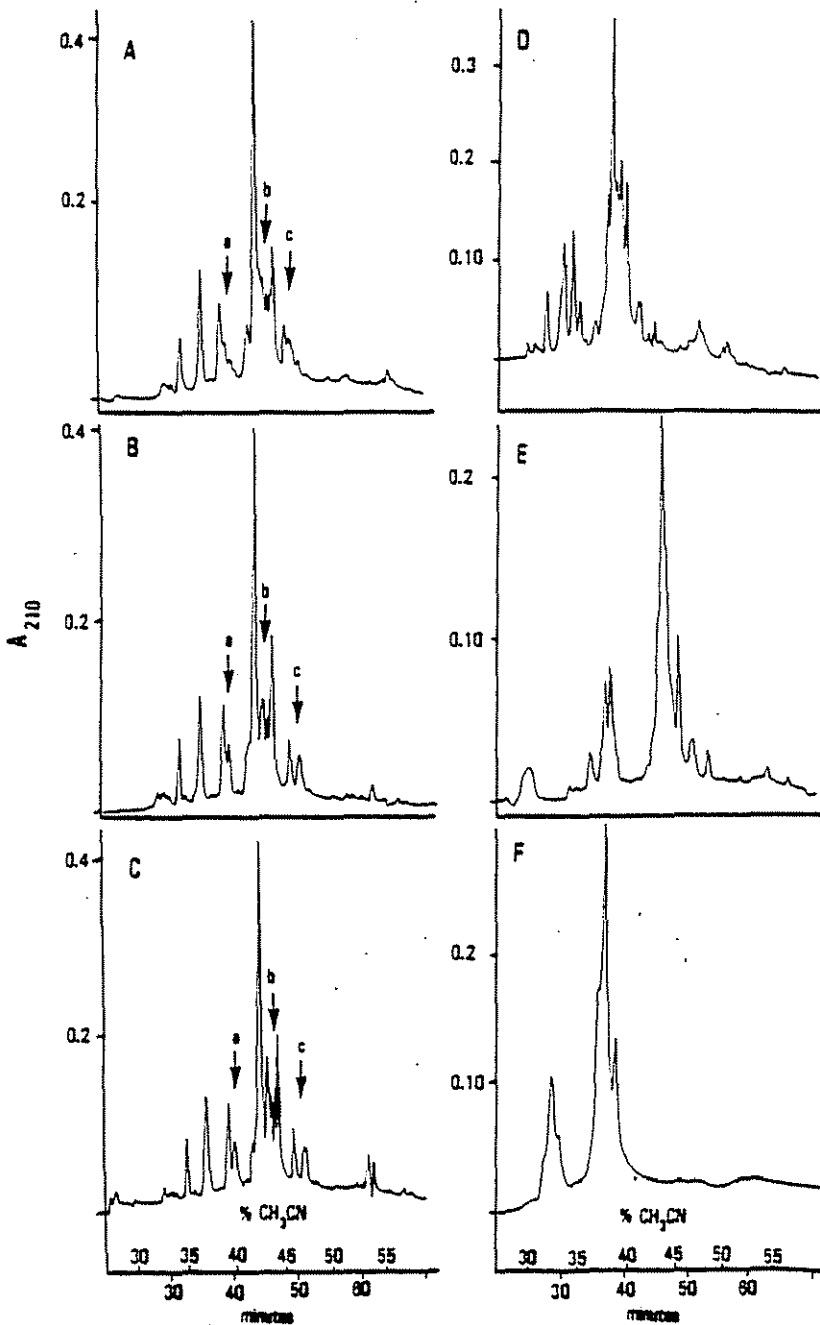
Figure 3. Reversed-phase high-performance liquid chromatography of pyridylethylated glutenin subunits on various columns: A, SynChropak RP-P (C18); B, Brownlee Aquapore RP-300 (C8); C, Bakerbond C8; D, Bakerbond diphenyl; E, Whatman diphenyl; F, Bakerbond cyanopropyl. Proteins were eluted with a linear gradient from 13 to 60% acetonitrile (+ 0.1% trifluoroacetic acid) during 65 minutes. Arrows a–c show peaks. (Reprinted, with permission, from Burnouf and Bietz, 1984b)

not be optimal for all columns. Obviously, however, the different selectivities of these columns may resolve samples difficult to separate under standard conditions.

F. Column and System Maintenance and Repair

With reasonable care and preventive maintenance, as specified by manufacturers, most HPLC equipment is relatively reliable and trouble free. Certain seals in pumps and injectors require periodic replacement, but this is infrequent when nonvolatile solvent components are washed from the system before the pumps are stopped. Chromatographic fittings may also leak, generally because of over- or under-tightening or incompatibility of male and female counterparts. Such leaks are detected by periodically measuring the flow rate at the detector outlet and by observing the system pressure.

The entire chromatographic system, with the exception of the column (which must be removed), can also be cleaned with nitric acid (generally 50%) if contamination is present or suspected; all parts in contact with solvents are stainless steel, Teflon, or other inert substances. Considerable care must be taken, however, to first wash the system extensively with water and to ensure that no blockage or leaks are present before the nitric acid is introduced, because of its highly corrosive nature. After a nitric acid wash, the system should be washed extensively with water to remove all nitric acid, which could damage the column and has high 210-nm absorbance.

Minimal care and preventive maintenance can also keep columns operating well and efficiently for long periods. After use, RP-HPLC columns should be washed with and stored in ACN or methanol to remove nonvolatile and/or acidic solvent components and to elute strongly retained solutes. Similarly, before reuse, columns should be washed with a solvent more hydrophobic than the final gradient conditions (e.g., 80–100% ACN + 0.1% TFA) to ensure that all peaks result from sample, rather than from solutes previously bound to the column. For this reason, a good procedure is to ignore the first run in any series, to run a blank first, or to analyze the first sample in duplicate.

In spite of these precautions, even with suitable guard columns and/or filters, the columns will eventually become contaminated, or pressures will increase. Since silica is very stable from pH 2 to 8, many solvents may be used for cleaning columns. ACN or 2-propanol elute most residual polypeptides, but urea or reducing agents may be included to solubilize HMW or cross-linked proteins. Lipids, if present, may be washed from the column with benzene, chloroform, and/or acetone, if care is taken to ensure solvent miscibility at each change. Aqueous solutions of dimethylsulfoxide (50–90%) are extremely useful for solubilizing and eluting strongly bound solutes or precipitated materials from RP-HPLC columns (Burnouf and Bietz, 1984b); such materials may include starch solubilized with urea solutions but subsequently insoluble in ACN solvents.

If such procedures do not reduce pressure or restore column performance, more drastic measures may succeed. Columns may generally be reversed to better remove particulates at inlet frits; although generally recommended by column manufacturers only as a last resort, this procedure usually does not harm the column. Column inlet fittings may also be disassembled and inlet frits

cleaned (by sonication in 50% nitric acid, followed by extensive washing with water) or replaced. Voids at the column inlet cannot be tolerated, since they lead to poor resolution or "double" peaks. Voids may sometimes be filled with additional packing or, with newer column types, eliminated by tightening the end fittings. It may also be possible to physically remove and replace contaminated packing near the inlet. If the voids cannot be repacked or the resolution restored, one should remember that nothing lasts forever.

III. RP-HPLC

Of the various HPLC techniques for proteins, RP-HPLC has been most widely used to fractionate and characterize cereal proteins. In part, this is because RP-HPLC is a powerful method that is complementary to those used previously. As discussed above, RP-HPLC has numerous other advantages. This section illustrates and describes RP-HPLC separations of wheat, maize, barley, oats, and sorghum proteins and illustrates applications of RP-HPLC in cereal protein chemistry.

A. Cereal Protein Separations

WHEAT STORAGE PROTEINS

In the brief history of RP-HPLC for analysis of cereal proteins, most studies have been of wheat. This section reviews typical separations of three classes of wheat proteins; additional examples of separations follow in the section on applications.

Gliadins. In the first study of cereal protein RP-HPLC (Bietz, 1983), variables were systematically explored and the applicability of RP-HPLC to all wheat protein classes was established. Initial (Bietz, 1983) and recent (Bietz, 1986) RP-HPLC separations of gliadin are compared in Figures 4A and 4B, respectively. In the original separation (Figure 4A) at 30° C, 36 peaks (including minor ones and shoulders) were observed; for the recent fractionation, performed in the same time, computer analysis actually revealed 88 components (including 24 minor peaks eluting earlier or later than the region shown). This enhanced resolution largely resulted from raising the column temperature to 70° C (see also Figure 1).

Such high resolution by RP-HPLC is certainly impressive. Gliadins are a heterogeneous multigene family, coded by genes at two complex loci in each of the three genomes of hexaploid wheat (Payne et al, 1984); they are thus a formidable challenge to any fractionation method. Twenty to 30 gliadin bands are usually resolved by one-dimensional electrophoresis (e.g., Bushuk and Zillman, 1978), whereas 46 were resolved by two-dimensional electrophoresis combining isoelectric focusing (IEF) and starch gel electrophoresis (Wrigley and Shepherd, 1973). Gliadin resolution by other two-dimensional procedures (Brown and Flavell, 1981; Jackson et al, 1983) may be better but still does not resolve all components. RP-HPLC also does not resolve all gliadins, but its resolution (even using short analysis times) is usually as good as that of one-dimensional electrophoresis and frequently matches that of two-dimensional procedures.

Glutenins. Fractionation of ethanol-soluble glutenin subunits was first

reported by Bietz (1983), and Burnouf and Bietz (1984b) later established optimal conditions for extraction, reduction, alkylation, and RP-HPLC analysis of all glutenin subunits. Glutenin may be dissociated with $8M$ urea or $6M$ guanidine hydrochloride, reduced with 5% ME or 0.1% dithiothreitol,
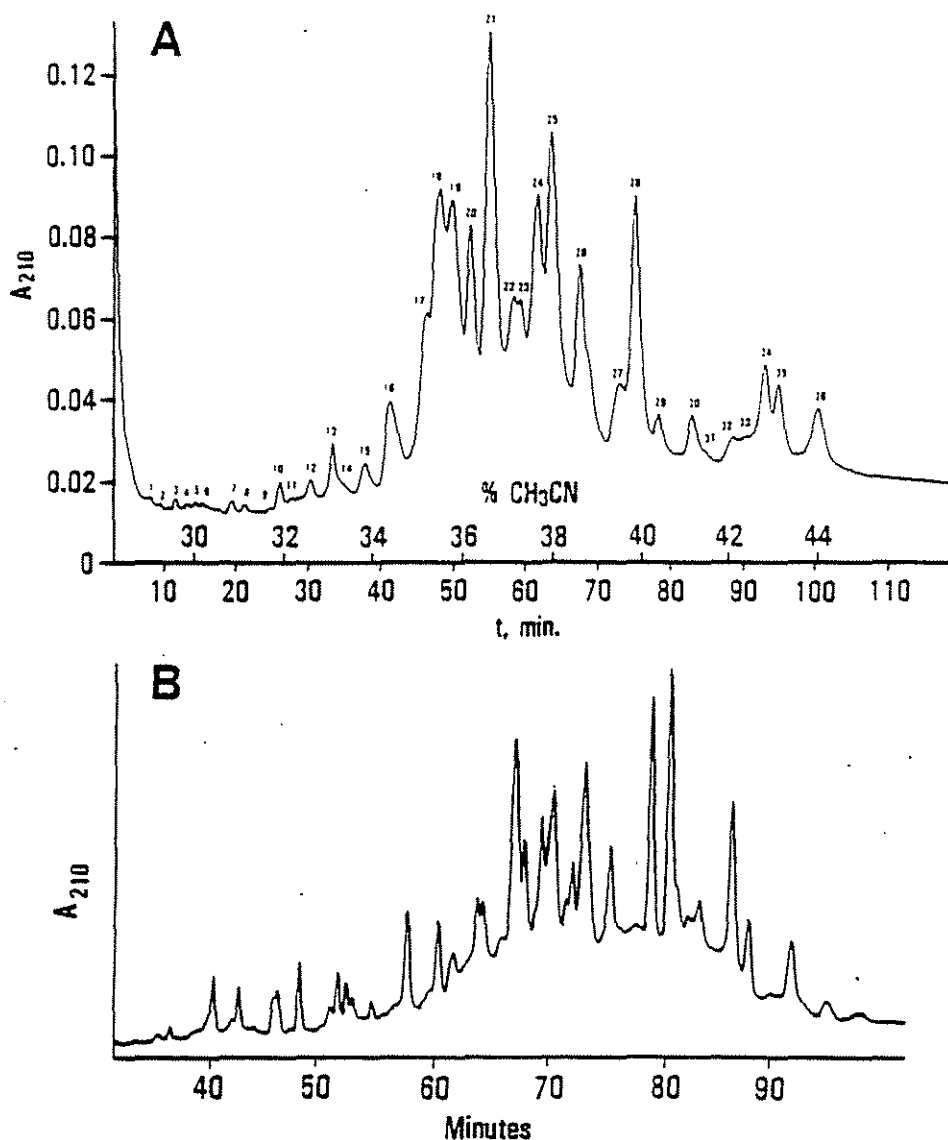

Figure 4. Fractionation of wheat gliadin by reversed-phase high-performance liquid chromatography. A, Ponca gliadin was separated on a SynChropak RP-P (4.1 × 250 mm) C18 column at 30° C using a gradient of 28–47.5% acetonitrile (+ 0.1% trifluoroacetic acid) during 120 minutes; numbers show peaks. B, Newton gliadin analyzed at 70° C on a SynChropak RP-P (C18) column, as in A, using a 15–55% acetonitrile (+ 0.1% trifluoroacetic acid) gradient during 120 minutes. (Reprinted, with permission, from Bietz, 1983 [A] and Bietz, 1986 [B])

alkylated with 4-vinylpyridine, and chromatographed on C18 or C8 columns, using an ACN gradient (Figure 5). At least 19 major peaks (plus several minor ones) resulted from RP-HPLC of Chinese Spring glutenin; early peaks (1–4) were identified as HMW glutenin subunits associated with breadmaking quality (Burnouf and Bietz, 1985), whereas later-eluting peaks contained ethanol-soluble subunits of lower molecular weight.

Albumins and Globulins. RP-HPLC also provides excellent resolution of wheat albumins and globulins (Figure 6). A 0.1*M* NaCl extract of Chinese Spring was separated on a C18 column using a gradient of 15–51% ACN. Approximately 46 components resulted (Figure 6a); computer expansion of raw data (Figure 6b), however, revealed approximately 86 major and minor components in the entire chromatogram. Frequently more than 100 peaks result upon RP-HPLC of complex mixtures, particularly at elevated temperature. RP-HPLC patterns of cereal albumins and globulins may change significantly
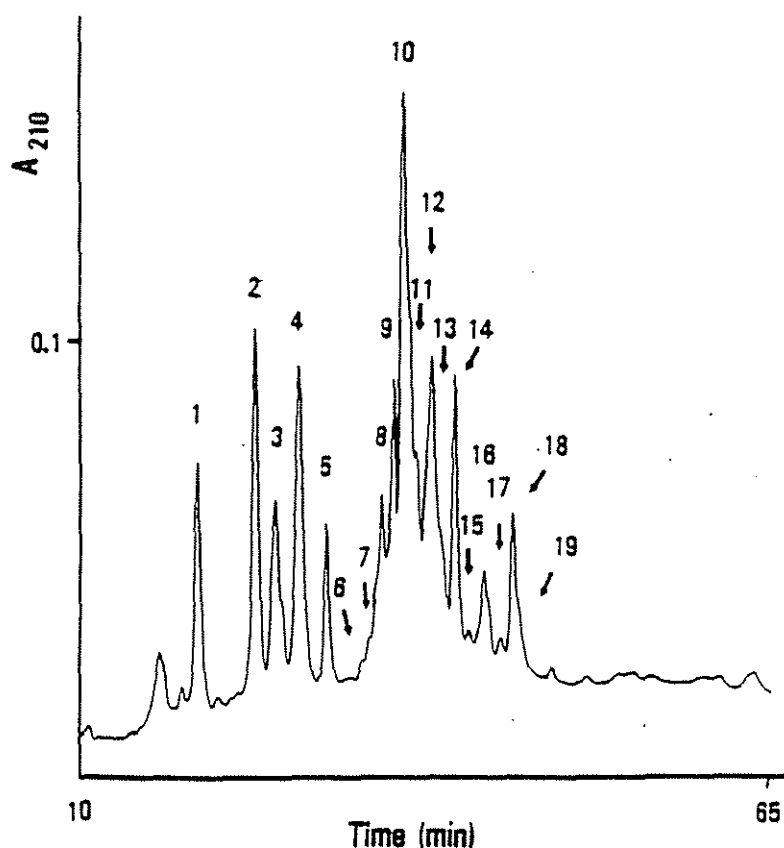
Figure 5. Reversed-phase high-performance liquid chromatography of reduced-alkylated subunits of Chinese Spring glutenin. Glutenin was reduced with 2-mercaptoethanol in the presence of 8 *M* urea, alkylated with 4-vinylpyridine, and chromatographed on a 250 × 4.1 mm Brownlee Aquapore RP-300 column (C8), using a gradient of 21–48% acetonitrile + 0.1% trifluoroacetic acid during 55 minutes at 1.0 ml/min. Numbers show peaks. (Reprinted, with permission, from Burnouf and Bietz, 1985)
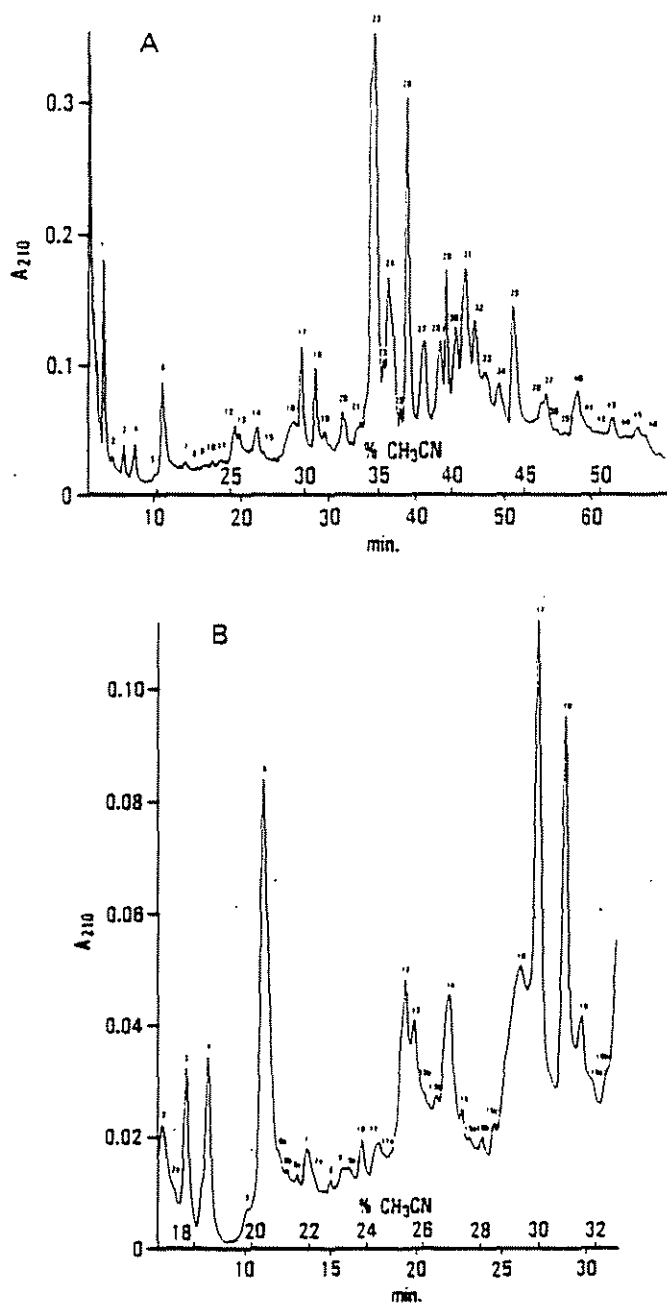
Figure 6. Reversed-phase high-performance liquid chromatography on SynChropak RP-P (C18) of albumin and globulin proteins extracted from a single kernel of Chinese Spring wheat with $0.1M$ NaCl. The column was eluted with a gradient of 15–51% acetonitrile (+ 0.1% trifluoroacetic acid) during 60 minutes. A, Entire chromatogram; B, computer-expanded portion of A, revealing additional minor peaks. Numbers show peaks. (Reprinted, with permission, from Bietz, 1983)

with time, however, possibly because of association through disulfide bonds or protease activity; thus, reproducible RP-HPLC fractionation of cereal albumins and globulins demands analysis under well-defined conditions, stabilization of extracted proteins (as, perhaps, through reduction and alkylation), or inactivation of endogenous proteases.

CORN STORAGE PROTEINS

RP-HPLC has also been used to isolate and characterize maize endosperm storage proteins. Zein from a normal hybrid was first resolved into 16 peaks eluting between 51.7 and 57.2% ACN (Bietz, 1983); these elution conditions are more hydrophobic than needed to elute most wheat proteins, agreeing with zein's hydrophobic composition and its limited solubility except in detergents, denaturants, or organic solvents. The resolution of zein was modified by substituting HFBA for TFA, thus modifying column selectivity (Bietz, 1983).

The preparative potential of RP-HPLC was also demonstrated for zein (Bietz, 1983). Zein (5.0 mg) was fractionated on an analytical column, giving resolution (24 peaks) nearly as good as that achieved with smaller samples. Characterization of fractions by IEF revealed considerable purification, although fractions were still heterogeneous, in accord with zein's known complexity (100 or more genes) (Hagen and Rubenstein, 1981). The power of RP-HPLC as an analytical and preparative method was apparent: zeins of identical surface hydrophobicity differed in charge, whereas other zeins with identical isoelectric points (and, presumably, molecular weight) varied in surface hydrophobicity. Thus, RP-HPLC complements zein separations based on size or charge, making it a valuable preparative method.

Zein RP-HPLC fractions have now been characterized by other methods (Paulis and Bietz, 1985). Native and reduced-alkylated zeins (25–50 mg) were fractionated on a semipreparative (10 mm i.d.) SynChropak RP-P (C18) column, and fractions were characterized by amino acid analysis, PAGE, SDS-PAGE, and IEF. Results revealed that zeins varying in molecular weight differed in the potential to polymerize through disulfide bonds; attempts were also made to relate surface hydrophobicity to hydrophobicity predicted from amino acid analysis.

Since cereal endosperm storage proteins accurately indicate genotype, RP-HPLC of zeins can differentiate and identify maize inbreds and hybrids (Bietz, 1985a), as can one- and two-dimensional electrophoresis (Wall et al, 1984). Zeins were extracted from ground single kernels with 1.0 ml of 70% ethanol and centrifuged, and extracts were analyzed at 70° C on wide-pore C18 columns, using a gradient of ~45–55% ACN (+ 0.1% TFA) during ~60 minutes. Most inbreds give significantly different patterns, permitting identification and estimation of genetic relationships. In addition, computer program CHROCP enables computer-derived and authentic hybrids to be compared in search of regulation of expression and may permit identification of parental lines of hybrids and F_2 populations.

RP-HPLC is now also used at other laboratories in maize breeding and genetic studies. Stephen Smith (Pioneer Co., Johnston, IA, personal communication) has found that HPLC provides a distinction between inbred lines that is equal to or better than that provided by isozyme analysis and that differences in HPLC patterns due to environment or year of seed production

account for no more than 5% of the observed variability. This agrees with the data of Paulis and Bietz (1986), who found by variance component analysis that the largest differences in relative peak areas among RP-HPLC chromatograms are due to genotype (81–95%); only 1–11% variance occurs among samples of the same genotype, and 4–7% variance is due to the analyses themselves. Thus, RP-HPLC can be used for maize genotype identification with high repeatability.

Paulis and Bietz (1986) also fractionated alcohol-soluble proteins other than zein by RP-HPLC. Zein, water-soluble alcohol-soluble reduced glutelin (WSASG, which is high in histidine and proline [Esen et al, 1982]), and water-insoluble alcohol-soluble reduced glutelin (WIASG, which is high in methionine [Esen et al, 1985]) were coextracted with a blend of 70% ethanol, 0.5% sodium acetate, and 5% ME. RP-HPLC (Figure 7) showed this extract to contain components of all three individual fractions. Most WSASG and WIASG subunits eluted earlier than did zeins, as was expected from their lower content of hydrophobic amino acids; some components of WIASG had elution times similar to those of zeins but were qualitatively and quantitatively different, demonstrating the complexity of maize glutelin and the unique nature of its alcohol-soluble subunits. Relative amounts and types of proteins in extracts varied significantly with genotype, permitting inbred identification, detection of mutant endosperm types, and prediction of lysine content. Thus, RP-HPLC provides a new method for determining maize protein quality.

BARLEY STORAGE PROTEINS

RP-HPLC also provides excellent separations of barley storage proteins (Figure 8). Marchylo and Kruger (1984) extracted hordeins with 50% 1-propanol plus reducing agent and separated them on a SynChropak RP-P (C18) wide-pore column using a gradient of 31–54% ACN (+ 0.1% TFA) during 105 (Figure 8, top) or 70 (Figure 8, bottom) minutes. Longer separations gave better resolution, which was judged to be important for differentiating some cultivars. Reduced extracts gave reproducible separations for three days; longer storage of extracts changed B hordein patterns, however, suggesting that reoxidation may occur. Alkylation with iodoacetamide had a deleterious effect on resolution and was not routinely used. Highly reproducible retention times were achieved when evaporation from solvents was controlled. Elution profiles of genotypes were independent of growth location, year of growth, and protein content, showing that RP-HPLC has considerable potential as a technique to identify barley cultivars.

Marchylo and Kruger (1985) recently extended these studies by using RP-HPLC to separate hordeins and identify cultivars of barley and barley malt. Three column types—Brownlee Aquapore RP-300 (C8), SynChropak RP-P (C18), and Ultrapore RPSC (C3)—gave somewhat different separations, but all were generally suitable. Optimal separations were obtained in 105 minutes, and resolution with a 50-minute gradient may often be satisfactory. Resolution was relatively poor with a 25-minute gradient but may be adequate for some purposes. Reproducibility was excellent: coefficients of variation ranged from 0.33 to 0.10%. The use of high temperature (70° C) decreased retention times and altered selectivity but did not significantly affect resolution; perhaps, in contrast to the findings for wheat (Bietz and Cobb, 1985), extraction under reducing conditions facilitates barley prolamin dissociation, so that improved resolution

at high temperature does not result. Chromatograms of malt hordeins were similar to those of barleys, but quantitative differences were observed. Nevertheless, all malted barley chromatograms were distinguishable, demonstrating the potential of RP-HPLC to identify barley malts. Marchylo and Kruger (1985) also demonstrated the power of computers to store raw data
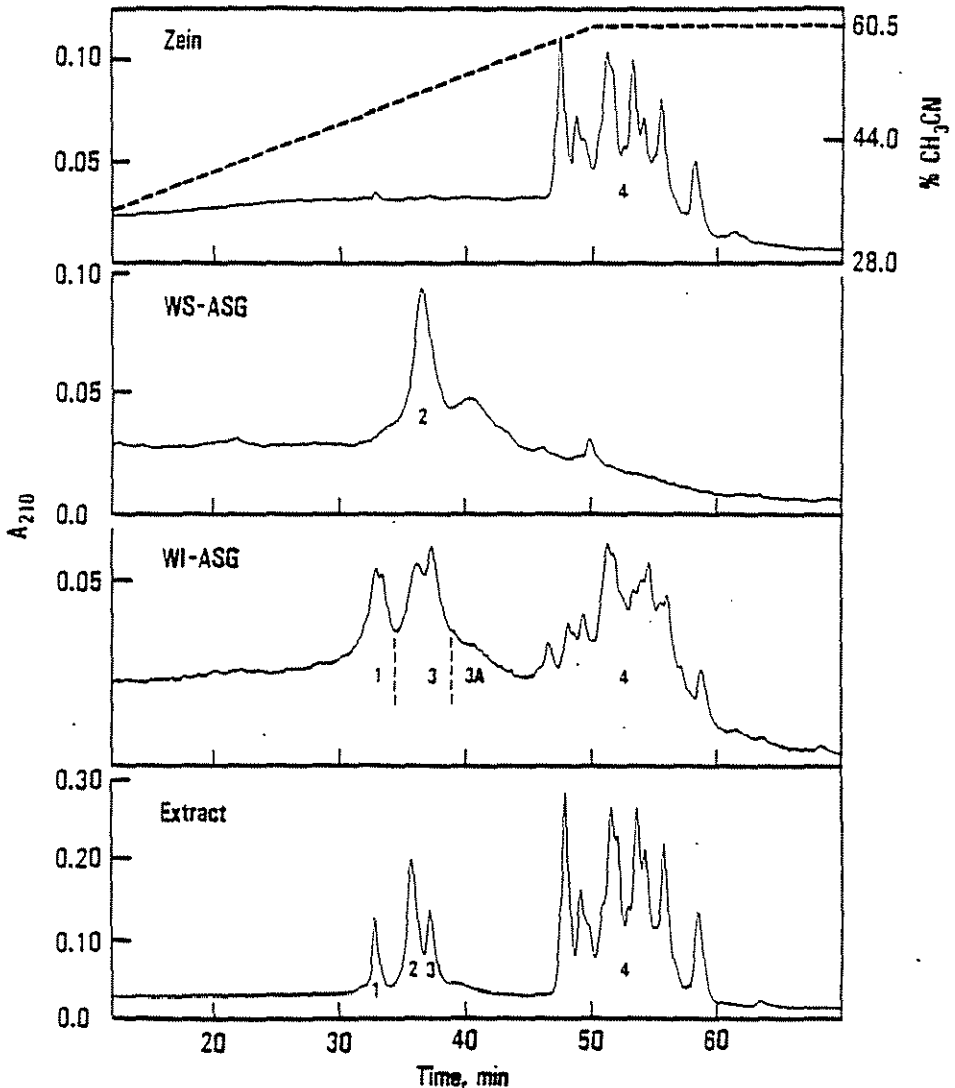

Figure 7. Reversed-phase high-performance liquid chromatography of endosperm proteins (soluble in 70% ethanol-0.5% NaOAc-5% 2-mercaptoethanol) from maize inbred W64A (extract) and of lyophilized and redissolved zein, water-soluble alcohol-soluble glutelin (WSASG), and water-insoluble alcohol-soluble glutelin (WIASG) from the same inbred. Chromatography was performed on a SynChropak RP-P (C18) 300-Å column, using a linear gradient of 28–60.5% acetonitrile (+ 0.1% trifluoroacetic acid). Numbers show comparable proteins. (Reprinted, with permission, from Paulis and Bietz, 1986)

for subsequent comparison and analysis: minor differences between chromatograms could be expanded and visualized, permitting differentiation of closely related cultivars.

OATS PROTEINS

Lookhart (1985a) recently demonstrated that RP-HPLC of oat prolamin, avenin (Figure 9), resolves at least three times as many components as does
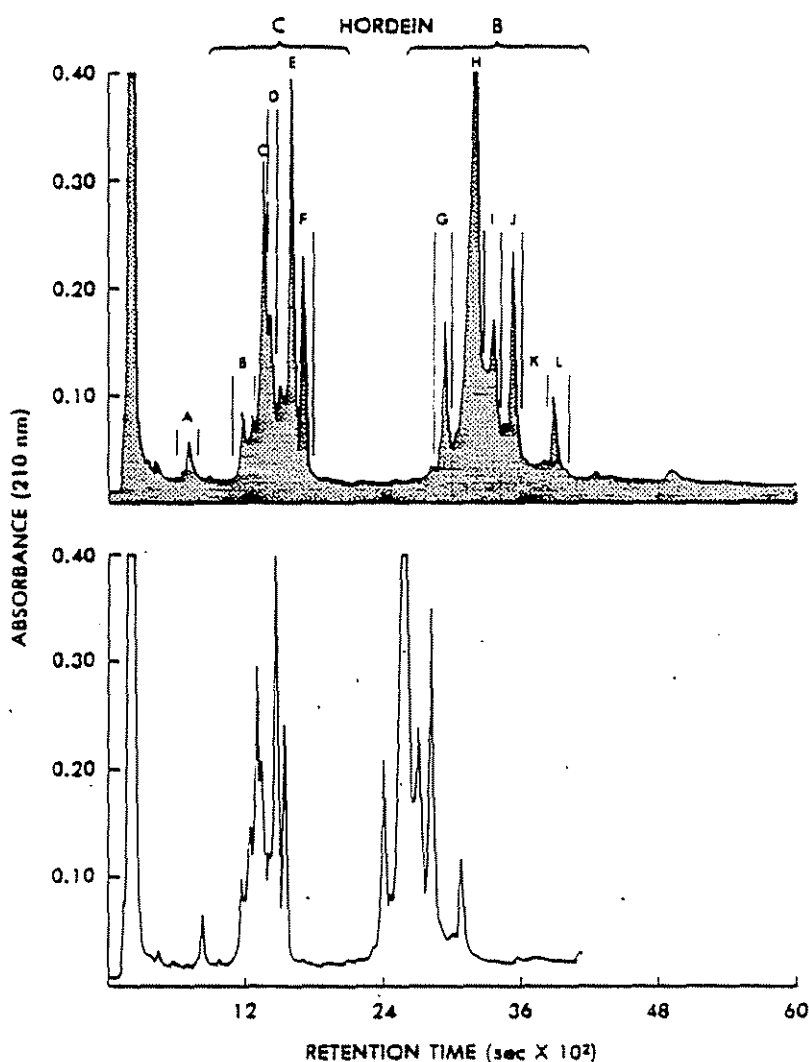

Figure 8. Reversed-phase high-performance liquid chromatography of hordein proteins extracted from the barley cultivar Betzes. Areas of hordein B and C proteins are shown. Chromatograms were developed at 1.0 ml/min, using a gradient of 31–54% acetonitrile (+ 0.1% trifluoroacetic acid) during 105 minutes (top) and 70 minutes (bottom). Peaks are shown by small letters. (Reprinted, with permission, from Marchylo and Kruger, 1984)

PAGE. Avenins were extracted with 70% ethanol and separated with a 32–56% ACN gradient. Chromatograms of different samples of the same cultivar were nearly identical, demonstrating the repeatability of RP-HPLC. Most cultivars, including those identical upon PAGE, could be differentiated, however, even by visual inspection. Thus, RP-HPLC was shown useful for oat prolamin characterization and cultivar identification. In another study, Lookhart and Pomeranz (1985a) used RP-HPLC and PAGE to characterize prolamins of major oat species. Pattern complexity increased with ploidy level. Combining RP-HPLC with PAGE was useful for characterizing prolamins of various species and may be useful for selecting variable genotypes for breeding programs.
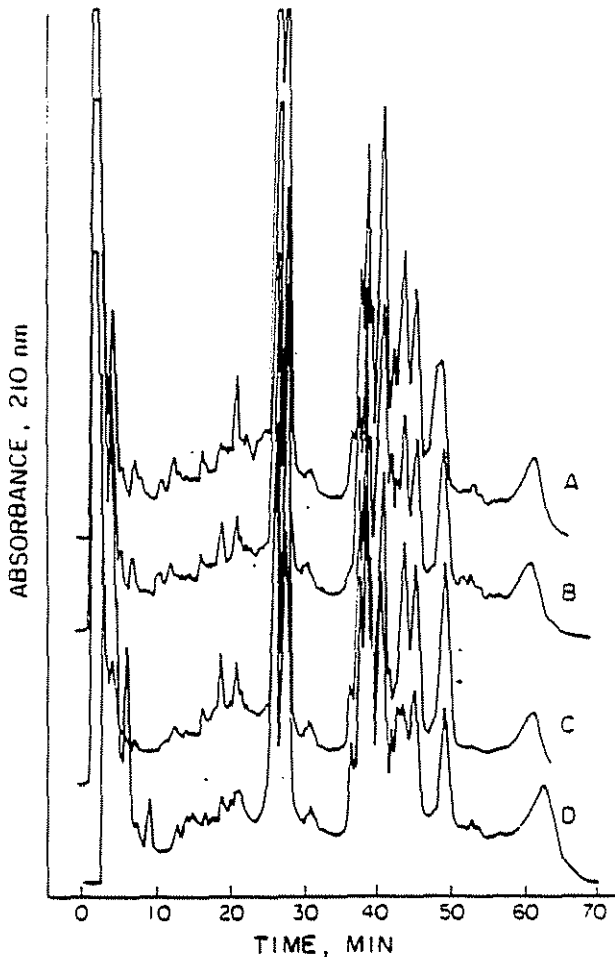
Figure 9. Reversed-phase high-performance liquid chromatography of avenin extracted with 70% ethanol from the oat cultivars Rodney (A), Harmon (B), Lang (C), and Kelsey (D). Chromatography was performed on SynChropak RP-P (C18) at 23° C, using a gradient of 32–56% acetonitrile (+ 0.1% trifluoroacetic acid) during 70 minutes. (Reprinted, with permission, from Lookhart, 1985a)

SORGHUM STORAGE PROTEINS

RP-HPLC, with IEF, has also demonstrated genetic variation among kafirins and alcohol-soluble glutelin (ASG) subunits from inbreds used to develop U.S. hybrids (Sastry et al, 1986a). Different inbreds contained different polypeptides, and hybrids contained proteins of both parents, with maternally contributed proteins predominating. Within a genotype, however, kafirin and ASG were similar qualitatively but differed quantitatively. RP-HPLC of alcohol-soluble proteins from sorghums of different races revealed that the greatest differences were between lines most widely removed from their center of origin. In a study of storage protein inheritance in high-lysine sorghums (Sastry et al, 1986b), RP-HPLC and IEF demonstrated that alcohol-soluble proteins from near-isogenic normal and high-lysine sorghums are qualitatively similar. Results also indicated that genes coding kafirins and ASGs were located on at least two chromosomes, one of which may also contain the *opaque* gene. RP-HPLC has also been used by Summers et al (1983) to fractionate kafirins for subsequent characterization.

PUROTHIONINS

A final example of RP-HPLC analysis of a unique cereal protein is work on purothionins (Jones et al, 1985). Many purothionins have similar molecular weight and charge, making isolation by IEC difficult. Consequently, RP-HPLC was used to separate wheat α-1, α-2, and β-purothionins. Up to 4 mg of purothionin mixture could be purified in one run (Figure 10). The elution order of α-purothionins (α-1 followed by α-2) is the reverse of that expected from their amino acid compositions, emphasizing that surface hydrophobicity does not necessarily correlate with total hydrophobicity.
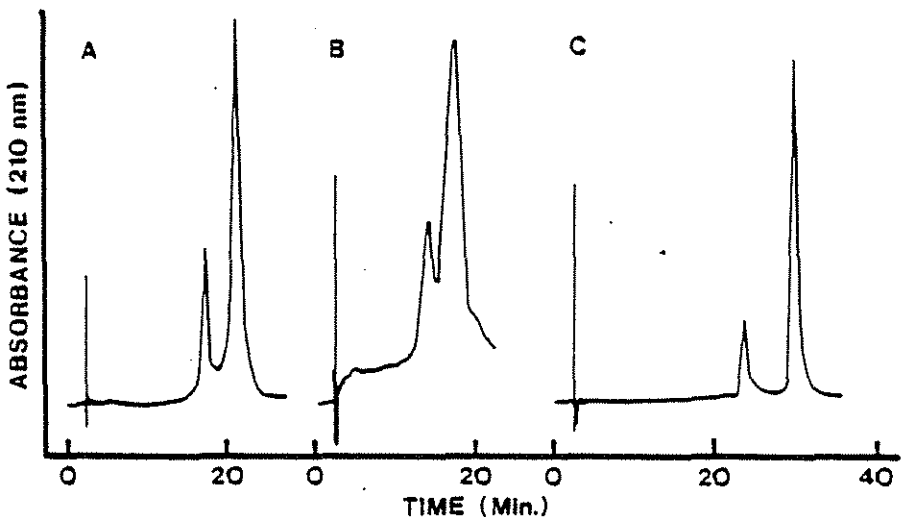

Figure 10. Effect of temperature (A, 20° C; B, 40° C; C, 0° C) on reversed-phase high-performance liquid chromatography (RP-HPLC) of α-purothionins. Purothionins were isolated from Manitou bread wheat flour and fractionated by RP-HPLC on SynChropak RP-P (C18), using a nonlinear gradient of about 25–31% acetonitrile (+ 0.1% trifluoroacetic acid) during 25 minutes. (Reprinted, with permission, from Jones et al, 1985)

B. Applications of RP-HPLC

CHARACTERIZATION OF SAMPLES

The examples in the previous sections illustrate separations of protein classes from many cereals, strongly suggesting that RP-HPLC is suitable for fractionation and characterization of all cereal (as well as noncereal) proteins. Homogeneity and heterogeneity are clearly demonstrated, and samples may be compared qualitatively and quantitatively. Since each endosperm protein type elutes largely as a unique group, Bietz and Burnouf (1985) suggested that protein type may actually be predicted from RP-HPLC elution characteristics and relative surface hydrophobicities. Comparison of a chromatogram of a reduced and alkylated (pyridylethylated) total protein extract of Centurk wheat (Figure 11) to chromatograms of isolated proteins indicated that polypeptides elute in the approximate order: 1) ω-gliadins, 2) HMW glutenin subunits, 3) LMW (α- and β-) gliadins, 4) LMW ethanol-soluble glutenin subunits, and 5) γ-gliadins. Some overlap occurs between adjacent areas, such as LMW gliadins and LMW glutenin subunits, but prediction of wheat protein type by RP-HPLC elution characteristics seems generally possible.
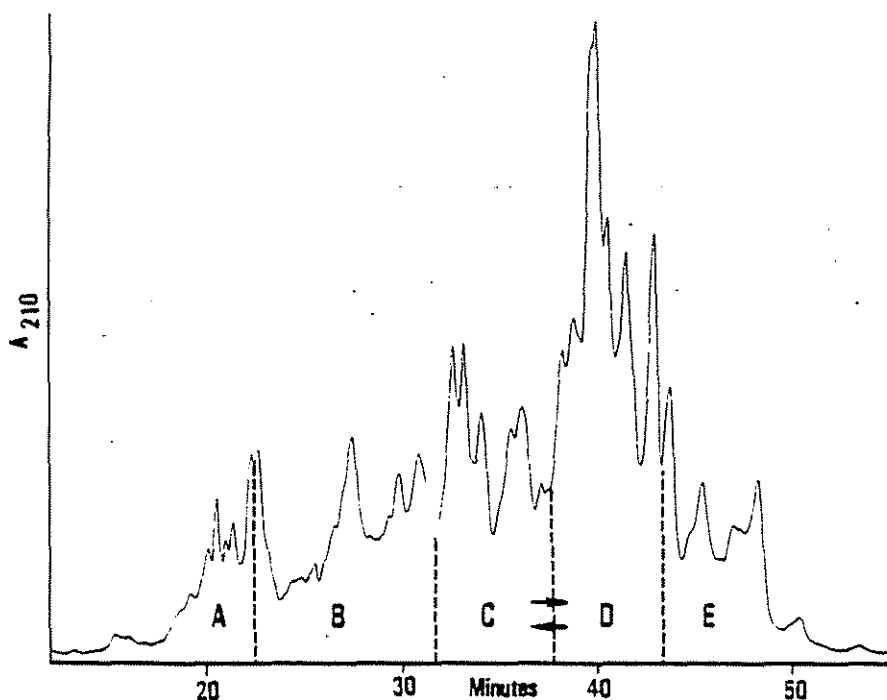

Figure 11. Reversed-phase high-performance liquid chromatography of a pyridylethylated total protein extract of Centurk wheat. A, ω-gliadins; B, high-molecular-weight glutenin subunits; C, low-molecular-weight (LMW) gliadins; D, LMW glutenin subunits; E, γ-gliadins. Proteins were fractionated on a Brownlee Aquapore RP-300 column at 70 °C, using a gradient of 21–48% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes.

PREPARATIVE APPLICATIONS

Since Bietz (1983) first showed that RP-HPLC, even on small diameter columns, can easily purify proteins in sufficient quantity for further use, several studies have already used this preparative capability.

RP-HPLC analysis of gliadins from durum wheat varieties revealed peaks thought to correspond to gliadins "45" and "42," which are associated with durum wheat quality (Damidaux et al, 1978). Ethanol-soluble proteins from 0.5 g of flour of two varieties were applied to a 250 × 10 mm (i.d.) column (Figure 12)
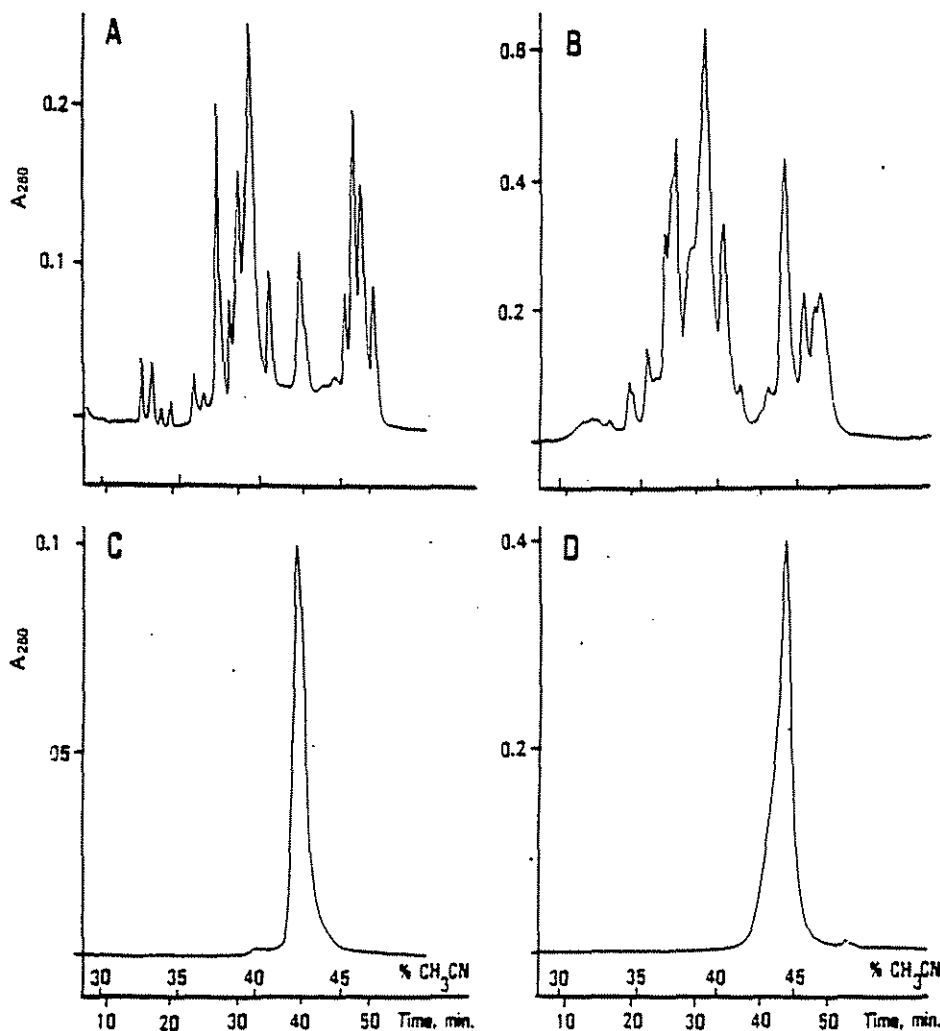
Figure 12. Preparative reversed-phase high-performance liquid chromatography on SynChropak RP-P (C18) (250 × 10 mm) of gliadins from the durum wheat varieties Edmore (A) and Langdon (B), and of collected chromatographic peaks corresponding to band "45" (C) and "42" (D). The column was developed at 3.0 ml/min with a gradient of 28–51% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes. (Reprinted, with permission, from Burnouf and Bietz, 1984a)

(Burnouf and Bietz, 1984a). The effluent was monitored at 280 nm to reduce absorbance, and fractions of interest were collected. Upon rechromatography, collected fractions exhibited single symmetrical peaks, shown by PAGE to be highly purified (though still not homogeneous) γ-gliadins 45 (Figure 12c) and 42 (Figure 12d). These components were thus easily and quickly (about one hour per run) prepared in quantities sufficient for further use, including antibody production for immunological studies of the structural similarity of gliadin to other cereal proteins (Burnouf and Bietz, unpublished data).

Burnouf and Bietz (1985) also used preparative RP-HPLC to isolate and identify the elution order of HMW wheat glutenin subunits. The first four peaks (as in Figure 5) were shown by SDS-PAGE to contain HMW glutenin subunits coded by chromosome arms 1DL, 1DL, 1BL, and 1BL (subunits 12 [1Dy], 2 [1Dx], 8 [1By], and 7 [1Bx], respectively, by the nomenclature of Payne et al [1981]). The heterogeneity of most other cereal protein fractions, however, seldom permits their complete isolation by RP-HPLC, since fractions homogeneous by surface hydrophobicity may still be heterogeneous by charge or size.

For this reason, Huebner and Bietz (1984) combined preparative RP-HPLC with classical IEC and gel filtration chromatography to purify gliadins. Extracts from wheat flour were first fractionated according to molecular weight on Sephadex G-100 and then by charge on sulfoethyl cellulose. Fractions were then subjected to preparative RP-HPLC, giving some nearly pure fractions. In many cases, however, fractions were still heterogeneous upon PAGE and IEF, demonstrating the heterogeneity (and possible deamidation) of gliadin and the necessity of using complementary methods to isolate individual polypeptides.

Preparative RP-HPLC is also valuable for isolation and characterization of corn prolamins (Paulis and Bietz, 1985). Native and reduced-alkylated zeins (30 mg) were separated on a SynChropak RP-P (C18) column (250 × 10 mm) by a gradient of 44–57% ACN during 120 minutes. Fifteen to 17 peaks resolved from each sample; this resolution is nearly comparable to that (at 30° C) for small samples on an analytical column. Fractions were characterized by amino acid analysis, IEF, PAGE, and SDS-PAGE, revealing zein's heterogeneity by charge, size, and surface hydrophobicity and suggesting that zein oligomers form preferentially from certain polypeptides.

As noted above, preparative RP-HPLC was also used to purify α- and β-purothionins (Jones et al, 1985). These several applications demonstrate that RP-HPLC is an extremely valuable preparative, as well as analytical, method. The speed of RP-HPLC, combined with automated sample injectors and fraction collectors, permits rapid and easy purification of polypeptides in quantities sufficient for further use.

QUANTITATION OF RP-HPLC DATA

Because RP-HPLC column effluents can be monitored at ≈210 nm, and because of the sensitivity and reproducibility of RP-HPLC, the quantification of data is superior to that of most other methods. At this wavelength, peptide bond carbonyl groups absorb UV light. Protein side chains (and nonprotein constituents) may also absorb at this wavelength, but their contribution is generally less than that of peptide bonds, so the absorbance is nearly proportional to the number of amino acid residues present. Some variability in

the absorbance of different proteins may be expected; the extent of this variability has not been determined but seems relatively small. Certainly, quantitation at 210 nm is superior to that at 280 nm, where absorbance is due primarily to tryptophan and tyrosine. Similarly, quantitation by 210-nm absorbance is preferable to staining with Coomassie blue for electrophoresis, since dyes bind primarily to basic amino acids, the content of which differs significantly among proteins. Significant applications for quantitating cereal protein RP-HPLC data are summarized in this section.

In studies of the chromosomal control of hexaploid wheat glutenin subunits, Burnouf and Bietz (1985) found that early-eluting RP-HPLC peaks (peaks 1–4 in Figure 5) contain subunits coded by genes on the long arms of chromosomes 1D and 1B; SDS-PAGE identified these as glutenin's HMW subunits, which are associated with breadmaking quality. Quantitation of RP-HPLC results (Figure 5) indicated that similar amounts of the two 1DL subunits (peaks 1 and 2) were present but that the 1BL subunits (peaks 3 and 4) differed in abundance. Such differences may indicate different numbers of structural genes. Thus, quantitation suggested that total gene copy numbers for HMW glutenin subunits on chromosome arms 1DL and 1BL are similar and that similar numbers of genes for two HMW subunits are present on 1DL, but unequal numbers of genes for two other HMW subunits are present on 1BL.

Huebner and Bietz (1985) subsequently found that the ratio of amounts of HMW to LMW glutenin subunits, as well as the presence or absence of specific HMW subunits, can also predict breadmaking quality. Since HMW glutenin subunits elute first upon RP-HPLC, their amount, relative to the total amount of glutenin, can be accurately estimated by integration. Comparison of these ratios for hexaploid wheats varying in baking quality revealed significant correlations between percentage of HMW glutenin subunits and mixing time ($r = 0.867$), loaf volume ($r = 0.658$), and general score ($r = 0.940$). Thus, RP-HPLC shows that the percentage of HMW glutenin subunits in wheat can be used to predict general score and mixing time but is less closely related to loaf volume.

Huebner and Bietz (1986) also recently found that quantitation of gliadin RP-HPLC data for wheats varying in breadmaking quality can predict quality. Visual examination of chromatograms of gliadins from varieties differing in breadmaking potential suggested an inverse relationship between amounts of late-eluting peaks and baking quality; these peaks correspond in hydrophobicity to those related to good and poor pasta quality in durum wheat varieties (Figure 12). Correlation coefficients of -0.71 to -0.95 were determined between relative gliadin chromatographic areas and general scores for wheats from several locations, suggesting that genes that code certain hydrophobic gliadins are either closely associated with genes that confer breadmaking quality to hexaploid wheat or are themselves partially responsible for such quality. The discovery of this relationship and its subsequent use obviously rely upon accurate quantitation of RP-HPLC data.

. Quantitation of RP-HPLC data has also been applied to maize proteins (Paulis and Bietz, 1986). Chromatograms of coextracted zeins and ASG subunits showed that areas corresponding to each protein class differed significantly for normal and mutant (*floury-2*, *opaque-2*, and *sugary-1*) genotypes. Maize alcohol-soluble protein content, estimated from the ratio of

total chromatographic area to the area of total protein, was also significantly negatively correlated ($r = -0.90$) with lysine content. This is similar to the inverse relationship found earlier between zein content (related to lysine), determined by Kjeldahl analysis, and turbidity (Paulis et al, 1974). Thus, quantitative RP-HPLC analysis of alcohol-soluble maize proteins can estimate lysine content, as well as identify genotypes.

VARIETAL IDENTIFICATION

Since RP-HPLC gives high resolution of prolamins, which are accurate genotypic indicators, it can identify most wheat varieties (Bietz, 1983). RP-HPLC is also useful for identification of varieties of other cereals and for other genotype analyses, as described in this section.

Wheat. After gliadin extraction and RP-HPLC conditions were optimized (Bietz et al, 1984a), significant qualitative and quantitative differences were found among most hard red winter, hard red spring, soft red winter, white, and durum wheats (Bietz et al, 1984b). Only varieties having nearly identical pedigrees may be impossible to differentiate. A typical example of identifying wheat varieties by RP-HPLC is shown in Figure 13. These four durum wheat chromatograms, and those of most other durum varieties examined, differ by numerous peaks; the differences between closely related cultivars were, as expected, slight. All durum varieties also had either peaks b–e (Figure 13, A and B) or i and j (Figure 13, C and D), which are associated with pasta quality (Burnouf and Bietz, 1984a; see also Figure 12 and subsequent discussions). Similar differences were detected among varieties of other wheat classes, and in some cases wheat class could be predicted (Burnouf et al, 1983).

In the original RP-HPLC studies of wheat varietal identification (Bietz et al, 1984b), 55-minute gradients were used, with a run time of 65 minutes and a 10-minute equilibration delay. Although rapid, compared to electrophoresis, which may require one to two days to identify varieties (Wrigley et al, 1982), RP-HPLC under these original conditions may not permit sufficient analyses (about 20 per day) for routine use. Bietz and Cobb (1985) found, however, that improved resolution of gliadins at high temperature (Figure 1) permits rapid differentiation of varieties (Figure 2). An example for 10 English wheats is shown in Figure 14. With an eight-minute gradient, 10-minute run time, and three-minute reequilibration, resolution was inferior to that of longer runs, but varieties could still be differentiated and identified. Using these conditions, one can now automatically analyze 100 samples per day; this high productivity suggests that RP-HPLC can be routinely used for varietal identification and for detection of inferior wheats (J. R. S. Ellis, personal communication). RP-HPLC, combined with rapid sample extraction and clarification methods, may also permit sample identification within 15 minutes of receipt, an advantage for purchasing and processing wheat or formulating decisions.

Using slightly different techniques, Kruger and Marchylo (1985a) "fingerprinted" Canadian wheat varieties by RP-HPLC of alcohol-soluble proteins. Optimal extraction was achieved using 50% 1-propanol, 1% acetic acid, and 4% dithiothreitol and a 105-minute gradient at 1.0 ml/min. The suitability of various RP-HPLC columns was also examined. RP-HPLC patterns for a variety grown at different locations and in different years were qualitatively the same; this similarity has also been demonstrated by
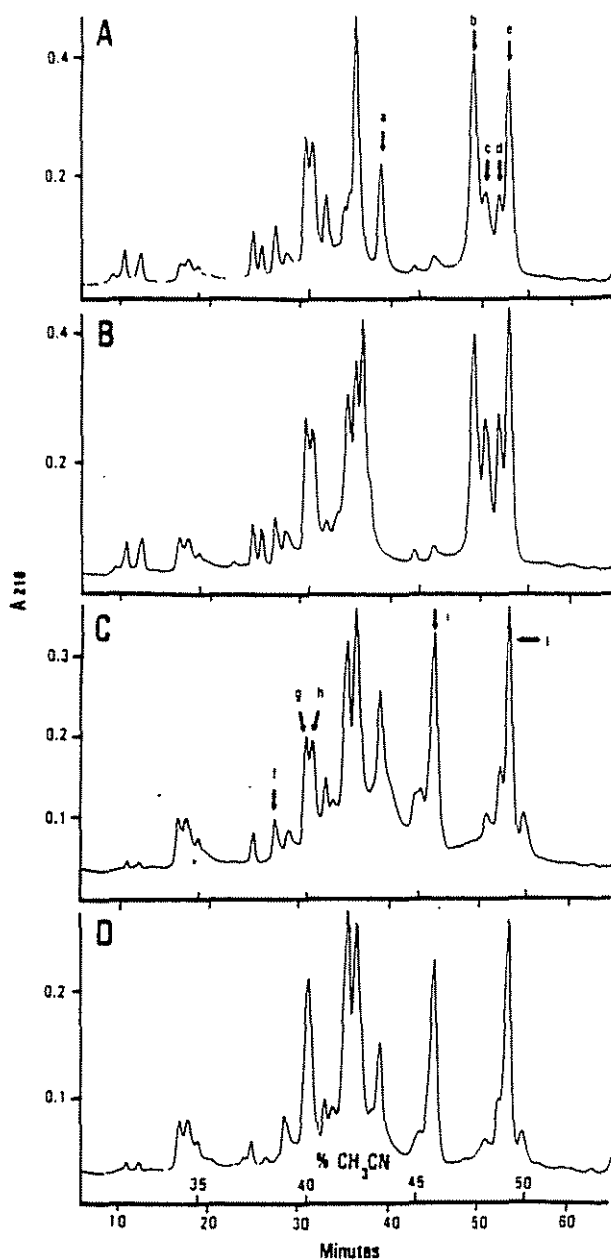
Figure 13. Reversed-phase high-performance liquid chromatography patterns of gliadins from the durum wheat varieties Ward (A), Rolette (B), Edmore (C), and Mexicali (D). Samples were chromatographed on SynChropak RP-P (C18), using a gradient of 28–51% acetonitrile (+ 0.1% trifluoroaceticacid) during 55 minutes. (Reprinted, with permission, from Bietz et al. 1984b)
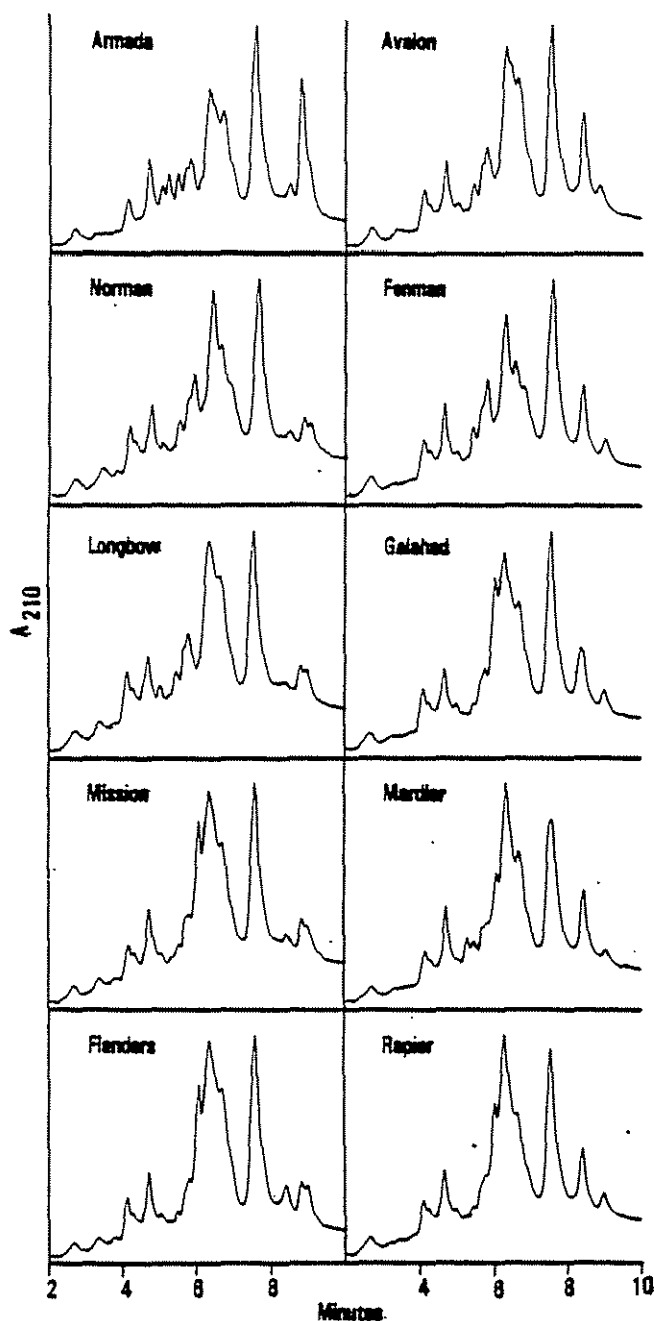
Figure 14. Rapid reversed-phase high-performance liquid chromatography of gliadins from 10 English wheat varieties. Samples were analyzed at 3 ml/min on a Brownlee Aquapore RP-300 (C8) column at 70° C, using a gradient of 25–50% acetonitrile (+ 0.1% trifluoroacetic acid) during eight minutes. (Reprinted, with permission, from Bietz and Cobb, 1985)

electrophoresis (Wrigley et al, 1982) and is an essential prerequisite for genotype identification through protein analysis.

Slight quantitative differences in storage polypeptide composition may occur within a genotype, however, particularly when varieties are grown under extremely different environmental or agronomic conditions; such variability could complicate varietal identification. This was demonstrated by electrophoresis, for example, for wheats grown on sulfur-deficient soils (Wrigley et al, 1984). Lookhart and Pomeranz (1985b) have now also shown that such changes may be detected by RP-HPLC, as well as by electrophoresis. RP-HPLC patterns of gliadins from sulfur-deficient wheats exhibited increased amounts of early-eluting peaks and lesser amounts of later-eluting gliadins; these results were attributed to the probable presence of increased amounts of HMW polypeptides in wheat grown on sulfur-deficient soil. Since early-eluting peaks observed upon RP-HPLC of gliadin have been identified as ω-gliadins (Bietz, 1983), it seems likely that the synthesis of ω-gliadins, which lack sulfur, increases upon severe sulfur deprivation, thus maximizing storage protein accumulation under these conditions.

Kruger and Marchylo (1985b) also examined wheat storage proteins during germination by RP-HPLC. Proteins extracted with 50% 1-propanol, 1% acetic acid, and 4% dithiothreitol appeared identical at any stage of germination, suggesting, based on the apparent lack of digestion products, that proteolysis during germination is extremely rapid. Thus, sprout damage of wheat should not complicate RP-HPLC cultivar identification.

Sapirstein and Bushuk (1985) recently developed techniques for computer identification of wheat varieties from gliadin PAGE data. Band mobilities and densities for unknown cultivars are compared to stored data for standards. These techniques, with modification, should also be applicable to RP-HPLC data, permitting computer-assisted identification of wheat varieties through RP-HPLC of gliadin. Since quantitation and reproducibility for RP-HPLC data may be superior to those of PAGE data, accuracy of RP-HPLC varietal identifications could improve. The subjective evaluation of component amounts can also be eliminated in RP-HPLC, and elution positions can be determined automatically. Thus, totally automated cultivar identification via RP-HPLC could soon be a reality.

Corn. Maize inbreds and hybrids may also be identified by RP-HPLC (Bietz, 1985b). Zeins extracted with 70% ethanol give reproducible high-resolution separations for most inbreds and hybrids (Figure 15), which reliably differentiate and thereby permit identification of genotypes. Thus, RP-HPLC should be useful in maize genetic and breeding studies. As with wheat, analysis time may be reduced significantly while reasonable resolution is retained (Figure 2).

Other Cereals. The procedures for RP-HPLC identification of wheat and maize genotypes also apply, with minor modification, to other cereals. Marchylo and Kruger (1984) differentiated all barley cultivars examined by qualitative and quantitative differences in the RP-HPLC elution patterns of extracted hordeins (Figure 8). Thus, RP-HPLC has considerable potential for barley cultivar identification. Similarly, Lookhart (1985a) showed that 23 major U.S. oat cultivars could be differentiated by combining PAGE and RP-HPLC (Figure 9). Sastry et al (1986a) noted that RP-HPLC is a valuable qualitative and quantitative method for resolving kafirins and ASGs of sorghum varieties

and races, providing a method complementary to electrophoresis for genotype identification. It seems likely that RP-HPLC procedures similar to those used to identify wheat, maize, barley, oats, and sorghum varieties will apply to all cereal grains.

SELECTION

In traditional plant breeding, genetic diversity must occur to permit improvement. Such diversity occurs in different genotypes, which can be accurately "fingerprinted" and selected by RP-HPLC. An example is Nap Hal, a hexaploid wheat variety having high protein and lysine character (Johnson et al, 1978). When gliadins of phenotypically different kernels from Nap Hal were examined by RP-HPLC (see Figure 7 of Bietz et al, 1984a), numerous qualitative and quantitative differences were detected. These results demonstrate that RP-HPLC can select potentially useful genotypes. Data also
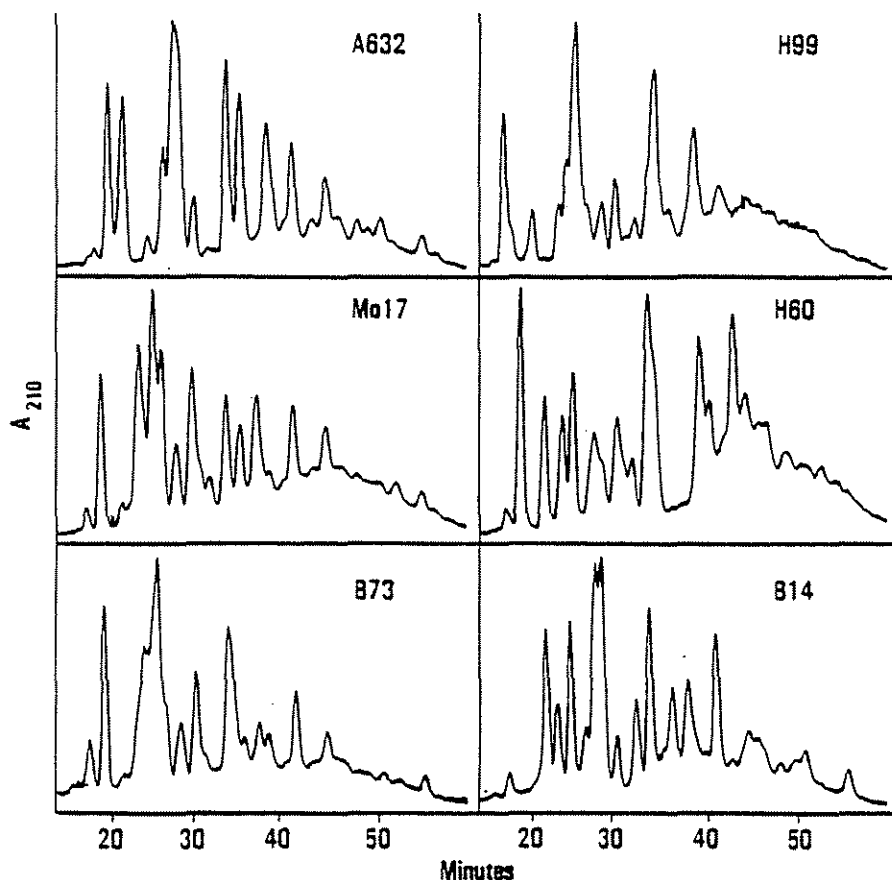

Figure 15. Identification by reversed-phase high-performance liquid chromatography of maize inbreds by zein analysis. Crushed endosperms of single kernels were extracted 30 minutes with 1.0 ml of 70% ethanol and chromatographed on a SynChropak RP-P (C18) analytical column at 70° C, using a gradient of 48–58% acetonitrile (+ 0.1% trifluoroacetic acid) during 60 minutes. (Reprinted, with permission, from Bietz, 1986)

suggest the desirability of authenticating varieties used in test crosses; even in homogeneous varieties from breeders and germ-plasm storage banks, up to 4% of the samples may be misidentified (Jones et al, 1982). These results also suggest that considerable caution must be used in applying single-kernel analysis, by either RP-HPLC or PAGE, to initial characterization of genotypes or to varietal identification. Numerous kernels or bulk samples should first be analyzed to establish standard patterns for any varieties.

Burnouf and Bietz (1984c) also demonstrated the value of RP-HPLC for detecting atypical genotypes in diploid and tetraploid *Triticum* and *Aegilops* species. Qualitative and quantitative variability was found among gliadin and glutenin proteins from accessions of *T. monococcum* and *T. dicoccum*. Such variability could be normal within a species, or may indicate sample misidentifications. RP-HPLC should be valuable for selecting such unusual accessions of a species for further characterization and for detecting genotypes having atypical characteristics from exotic germ-plasm sources that could be introduced into commercial cultivars.

Examination of Genetic Purity. Methods that can accurately and rapidly determine genotypic purity are increasingly needed. For example, in production and sales of hybrid seed corn, homogeneity is demonstrated by isozyme electrophoresis. Sensitive protein analytical methods have also shown that methods that detect heterogeneity by phenotypic seed or plant characteristics may no longer be valid; many "varieties" today are somewhat heterogeneous. U.S. wheat breeders in years past generally introduced quite homogeneous varieties, although some heterogeneity ("biotypes," "off-types," or spontaneous aneuploids) may always be present. Biotypes may be more prevalent if different breeding strategies are used (Dal Belin Peruffo and Pogna, 1982). Of course, numerous valid reasons to change breeding methods exist. Time to introduce a variety may decrease, and multilines have advantages. Wide crosses can also introduce desirable characteristics. If such strategies produce heterogeneous phenotypes or grains that are difficult to classify or market, however, they should change. These considerations make sensitive methods of examining varietal purity increasingly important.

PAGE of gliadins from recent U.S. wheat varieties, including Newton and Arkan (which is a cross between Arthur, a soft wheat, and Sage, a hard red winter variety [Martin et al, 1983]), indicated significant kernel-to-kernel and location variability within these varieties (Lookhart, 1985b; Lookhart et al, 1985). Similar variability can be detected by gliadin RP-HPLC (Bietz, 1984d; Bietz and Cobb, 1985) (Figure 16). In this example, two kernels of Arkan having different phenotypes were examined by normal and rapid RP-HPLC. Normal analyses showed differences between the kernels at $\approx$21 and 33–37 minutes; these differences were evident at $\approx$4.3 and 6.4 minutes in the rapid analyses. Thus, RP-HPLC of proteins from phenotypically different grains can reveal varying genotypes within varieties. Using rapid analyses, more than 100 samples may be analyzed per day, as demonstrated for Newton (Bietz and Cobb, 1985).

Registration of Germ Plasms. Since RP-HPLC is an alternative method for fingerprinting genotypes, considerable interest has been shown in its use for registering newly released and proprietary germ plasms and varieties. As analytical conditions become more standard and the variability of RP-HPLC becomes better defined, RP-HPLC should find increasing use in this area.

GENETIC APPLICATIONS OF RP-HPLC

Bietz (1983) first noted that RP-HPLC can demonstrate chromosomal control of cereal proteins through aneuploid analysis. Analysis of gliadins from Chinese Spring wheat and two ditelocentric lines (lacking a pair of chromosome arms) subsequently showed that proteins coded by genes on the missing chromosome arms were absent in the aneuploids (see Figure 6 in Bietz et al, 1984b). Similarly, the chromosomal locations of genes that code HMW wheat glutenin subunits were determined by RP-HPLC analysis of nullisomic-tetrasomic (lacking one chromosome pair but having an extra pair of homoeologous chromosomes) and ditelocentric aneuploid lines (see Figure 6 in Burnouf and Bietz, 1984b). Recent detailed studies have now more fully characterized the gliadin and glutenin polypeptide loci in hexaploid and tetraploid wheats (Bietz and Burnouf, 1985; Burnouf and Bietz, 1985).

Gliadins of Chinese Spring hexaploid wheat and its nullisomic-tetrasomic and ditelocentric lines were analyzed by RP-HPLC (Bietz and Burnouf, 1985). Thirty-five peaks resulted for Chinese Spring; by observing which peaks were missing in aneuploid lines, researchers found that all gliadins were controlled by genes on the short arms of the group 1 and 6 chromosomes (the complex *Gli-1* and *Gli-2* loci), confirming the results of electrophoresis. Gluten polypeptides generally eluted as groups in the order: 1) albumins + globulins, 2) ω-gliadins, 3) HMW glutenin subunits, 4) α-type gliadins, 5) LMW glutenin subunits, and 6) γ-gliadins (see Figure 11 for a similar representation of proteins in Centurk). This was determined, in part, by observing that early gliadin peaks were coded
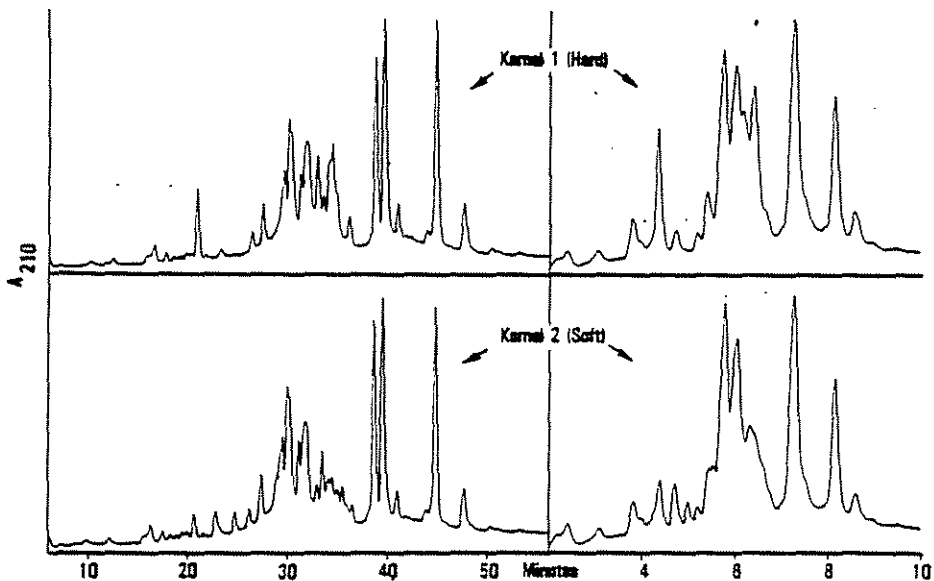

Figure 16. Analysis of two phenotypically different kernels (top, hard; bottom, soft) selected from a bulk sample of the hard red winter wheat variety Arkan. Reversed-phase high-performance liquid chromatography was performed by standard (left) and rapid (right) analyses of extracted gliadins on a Brownlee Aquapore RP-300 (C8) column at 70° C, using a gradient of 25–50% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes (at 1.0 ml/ min) or eight minutes (at 3.0 ml/ min). (Reprinted, with permission, from Bietz and Cobb, 1985)

by genes on the short arms of chromosomes 1A, 1B, and 1D (the *Gli-1* loci, which Bietz [1983] previously showed to be ω-gliadins); gliadin peaks of intermediate hydrophobicity were coded by genes on the short arms of chromosomes 6A, 6B, and 6D (the *Gli-2* loci) and were thus α-type LMW gliadins; and late-eluting hydrophobic gliadins were controlled by genes on the short arms of chromosomes 1A, 1B, and 1D (the *Gli-1* loci) and were thus γ-gliadins (see also Burnouf and Bietz, 1984a). Also, the three protein types coded by genes at the complex *Gli-1* loci (ω-gliadins, γ-gliadins, and LMW glutenin subunits) have uniquely different surface hydrophobicities.

Gene locations for gliadins of hexaploid Cheyenne wheat and the durum varieties Langdon, Edmore, and Kharkovskaya-5 were also determined by RP-HPLC of substitution and addition lines. Known locations of genes for gliadins were confirmed, again demonstrating the power of RP-HPLC in genetic studies by establishing markers for specific chromosome arms.

Glutenin subunits from Chinese Spring aneuploids and from substitution lines of the durum variety Langdon have also been examined by RP-HPLC (Burnouf and Bietz, 1985). Of the, 19 major peaks in Chinese Spring (Figure 5), polypeptides in peaks 1–4 were controlled by genes on the long arms of chromosomes 1D (peaks 1–2) and 1B (peaks 3–4). SDS-PAGE showed that these peaks contained glutenin's HMW subunits associated with breadmaking quality, eluting in the order 1Dy, 1Dx, 1By, and 1Bx, respectively. Late-eluting Chinese Spring glutenin subunits (Figure 5) were coded by structural genes on the short arms of homoeologous group 1 chromosomes (the complex *Gli-1* loci). Analysis of Langdon, Edmore, and Kharkovskaya-5 durum substitution lines also revealed gene locations of many durum glutenin subunits. These studies show that RP-HPLC is a powerful tool for qualitative and quantitative genetic studies of wheat glutenin and gliadin.

PREDICTION OF QUALITY

One of the most exciting applications of RP-HPLC is its use to identify quality-related proteins and, through their analysis, to predict quality. Examples of this, noted previously, are discussed here in more detail, along with the application of computers to quality prediction.

Burnouf and Bietz (1984a) first noted that RP-HPLC can predict durum wheat quality. Specific hydrophobic gliadins (Figure 13) divided durum varieties into two groups, corresponding exactly to varieties having PAGE bands 45 or 42, which are related to good and poor pasta quality, respectively (Damidaux et al, 1978). Thus, RP-HPLC, like PAGE, can screen for durum gluten quality. A rapid (200 analyses per day) gradient RP-HPLC procedure was also developed to screen for varieties with unacceptable glutens. Most gliadins eluted in the solvent peak, but peaks corresponding to bands 45 and 42 (see also Figure 12) were resolved. Because of its convenience and rapidity, RP-HPLC is a valuable alternative to PAGE in screening for durum quality in early generations of breeding.

Bread wheat quality can also be predicted by RP-HPLC. Burnouf and Bietz (1984b) found that HMW glutenin subunits, associated with breadmaking quality, elute first upon RP-HPLC (Figure 5, peaks 1–4). These subunits vary among cultivars (Burnouf and Bouriquet, 1980), permitting quality prediction by SDS-PAGE. This variability is also apparent upon RP-HPLC (Figure 17).
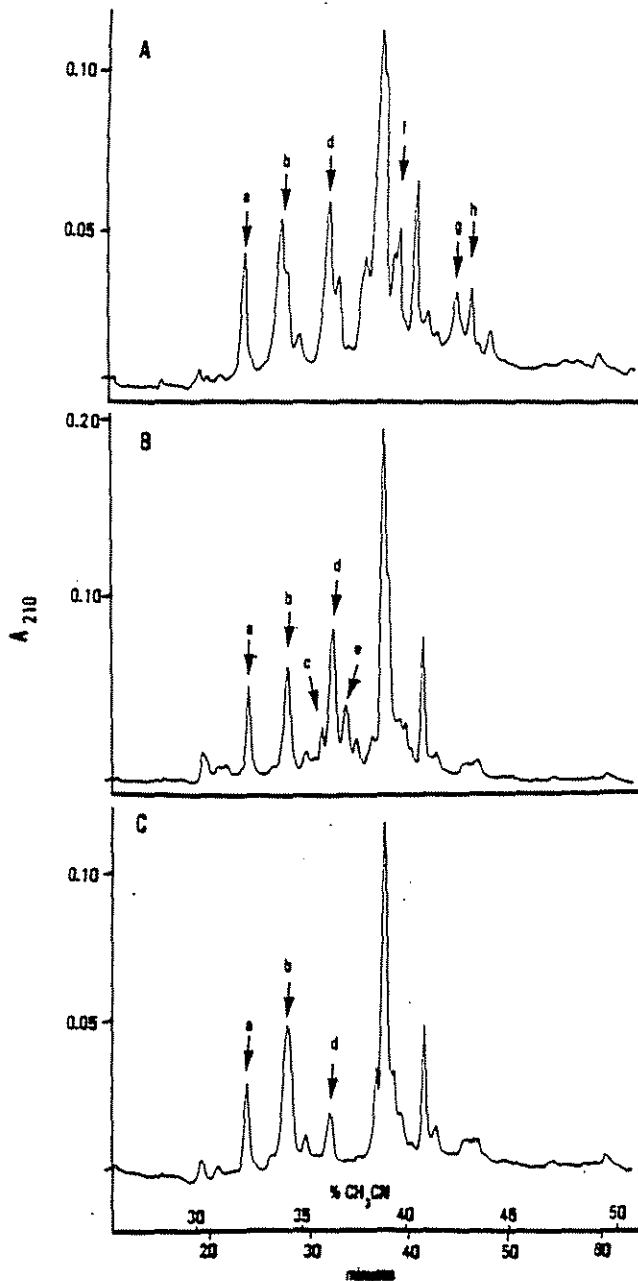
Figure 17. Reversed-phase high-performance liquid chromatography (RP-HPLC) of pyridylethylated glutenin subunits from the bread wheat varieties Marquis (A), Hardi (B), and Maris-Huntsman (C). Arrows indicate chromatographic peaks differing significantly among samples. RP-HPLC was performed on a Brownlee Aquapore RP-300 (C8) column, using a gradient of 22–53% acetonitrile (+ 0.1% trifluoroacetic acid) during 65 minutes. (Reprinted, with permission, from Burnouf and Bietz. 1984b)

Differences among·HMW glutenin subunit peaks a–e for cultivars differing in breadmaking quality may be related to the technological properties of the flour. RP-HPLC reveals similar differences among HMW glutenin subunits of standard varieties (Payne and Lawrence, 1983) typifying known alleles at the *Glu-A1, Glu-B1*, and *Glu-D1* loci, showing that RP-HPLC can generally predict wheat quality through glutenin subunit analysis (Burnouf and Bietz, unpublished observations).

Relative amounts of HMW and LMW glutenin subunits, determined by quantitative RP-HPLC, can also predict general score and mixing time, indicators of breadmaking quality (Huebner and Bietz, 1985). Relationships were previously demonstrated between molecular weight distributions of native glutenin and mixing time (Huebner and Wall, 1976), but the results of Huebner and Bietz (1985) are among the first to demonstrate that relative amounts of HMW and LMW glutenin subunits relate to mixing time and bread quality.

Glutenins are known to be closely related to breadmaking quality; some reports now also suggest that gliadins may also relate to quality. As noted above, Huebner and Bietz (1986) recently used RP-HPLC of gliadin to demonstrate such a relationship.

Similarly, as discussed above, quantitative RP-HPLC can predict the lysine content and nutritional value of maize. A correlation coefficient of −0.90 was found between the total peak absorbance per gram of protein and the percentage of lysine in protein (Paulis and Bietz, 1986).

Computer analyses should reveal additional quality factors. It should be possible, for example, to correlate the presence and size of peaks in numerous samples with measured quality factors, thus identifying components related to quality. Alternatively, the detector signal at each time interval could be correlated with measured characteristics; this procedure would eliminate integration and would be largely independent of normal variability in elution times or peak area. Correlation coefficients approaching +1 or −1 would indicate components related to quality, which could be isolated and further characterized or for which rapid tests could be devised. These examples suggest that RP-HPLC will become a general predictive tool for quality.

STUDY OF PROTEIN INTERACTIONS

RP-HPLC is also a powerful tool for studying association, cross-linking, conformation, and denaturation of cereal proteins and their interactions with other endosperm constituents. An example (Figure 18) shows 15 and 70° C chromatograms of isolated zein redissolved in 70% ethanol for RP-HPLC. At near-ambient temperature, resolution was poor. At 70° C, however, resolution was as good as for a fresh sample, and most components eluted later than at 15° C. It seems likely that these zeins had aggregated tenaciously upon dehydration and did not fully dissociate to their initial state when redissolved. The elevated column temperature apparently disrupted hydrogen or other noncovalent bonds (Bietz and Cobb, 1985), leading to complete dissociation.

RP-HPLC may, with other methods, also reveal how oligomeric proteins form. Paulis and Bietz (1985) found, by preparative RP-HPLC of native zein and examination of fractions by SDS-PAGE, PAGE, IEF, and amino acid analysis, that ordered association of certain zeins into dimers, trimers, and higher oligomers seems to occur. Reduced-alkylated zeins, however, do not

form oligomers, suggesting that disulfide bonds are involved in protein association.

RP-HPLC can also indicate changes in conformation or association of proteins that occur upon reduction of disulfide bonds. If, for example, one peak changes upon reduction to two or more components having different elution times, an oligomer stabilized by intermolecular disulfide bonds is indicated. If mobilities but not peak number are affected, intramolecular disulfides are indicated. If no changes in peak number or position occur upon reduction, disulfides are probably absent.
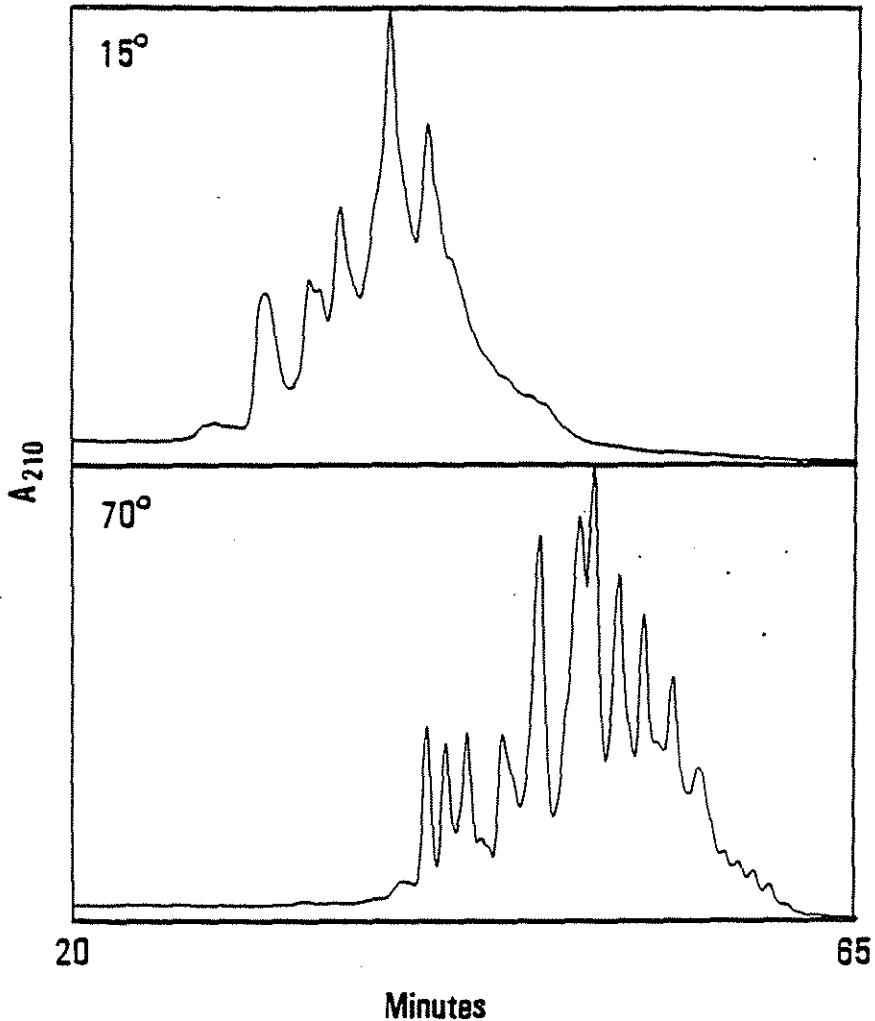

Figure 18. Reversed-phase high-performance liquid chromatography of 70% ethanol-soluble zein from maize inbred W64A on a Brownlee Aquapore RP-300 (C8) column at 15 and 70° C. The sample, which had been dialyzed and lyophilized, was redissolved in 70% ethanol and separated, using a gradient of 40–55% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes, with a run time of 65 minutes, at 1.0 ml/min. (Reprinted, with permission, from Bietz and Cobb, 1985)

Conformational changes resulting from cysteine modification may also be apparent. Most gliadins from Aldura durum elute earlier after reduction and pyridylethylation than when just reduced (Figure 19), due to the direct or indirect influence of alkylation on surface hydrophobicity or conformation. Thus, cysteine is clearly indicated in these gliadins. Similarly, differences between native and reduced gliadins (data not shown) demonstrate that
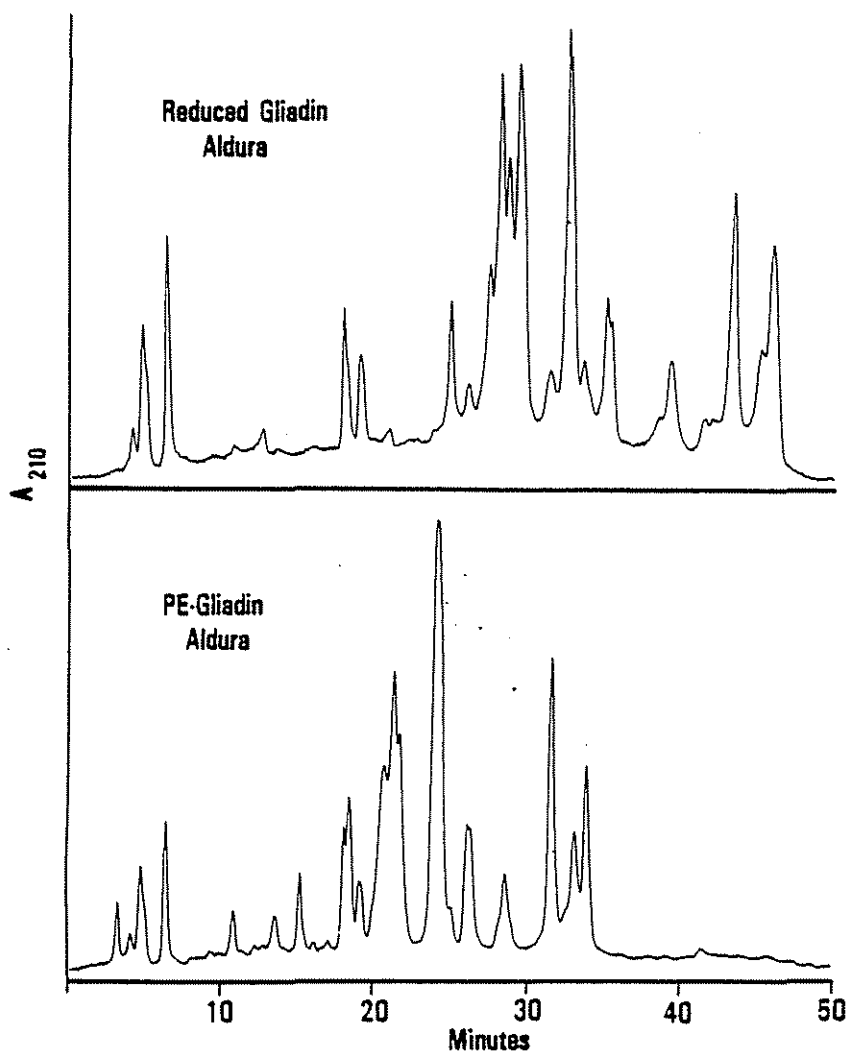

Figure 19. Effect of alkylation of wheat proteins on reversed-phase high-performance liquid chromatography elution time. Gliadin from the durum wheat cultivar Aldura, either reduced or reduced and pyridylethylated (PE), was chromatographed at 1.0 ml/min on a SynChropak RP-P (C18) column at 70° C, using a gradient of 23–50% acetonitrile (+ 0.1% trifluoroacetic acid) during 55 minutes.

disulfides exist in native gliadins. Approximately the same number of components is present in reduced, reduced-alkylated, and native gliadins, suggesting that most disulfides are intramolecular. Retention times of peaks before 10 minutes do not vary upon alkylation, however, suggesting the absence of cysteine; these are presumably ω-gliadins, which lack cysteine (Bietz, 1983). Similar changes occur between reduced and reduced-alkylated subunits of glutenin (see Figs. 1-3 in Burnouf and Bietz, 1984b), showing the effects of cysteine alkylation on surface hydrophobicity and presumably on conformation. Thus, RP-HPLC can provide information on disulfide bonding, as well as reveal association and conformational changes of cereal proteins.

Since surface hydrophobicity is related to protein structures, conformations, and functionality, RP-HPLC may reveal damaged or denatured proteins. For example, gliadins from immature wheat dried at high temperature were poorly resolved upon RP-HPLC (unpublished observations from this laboratory); similar studies might indicate gluten "vitality," which can be lost upon high-temperature drying (Schofield et al, 1983). Also, as indicated above, interactions of cereal proteins can be revealed by varying the column temperature in RP-HPLC.

Interactions with nonprotein constituents, such as lipids, should also be detectable. For example, Zawistowska et al (1985) characterized LMW wheat proteins with high affinity for flour lipids by RP-HPLC. Patterns for proteins from wheats differing widely in breadmaking quality differed; defatting of flour affected the sizes of the resulting peaks, indicating components that interact with lipids.

ANALYSIS OF HYBRIDS AND MIXTURES

As described above, computer programs such as CHROCP permit addition and subtraction of chromatograms. One promising application of this capability is analysis of hybrids or mixtures (Bietz, 1986). Total chromatographic areas were determined by integration for zeins from maize inbreds A632 and H60 (Figure 20); areas were normalized; and chromatograms were added; using the manual mode of CHROCP in a 2:1 ratio, corresponding to relative contributions of maternal and paternal genes to the triploid endosperm. The resulting computer-derived pattern was similar to that of zein from the hybrid A632 × H60, confirming its pedigree, the mode of zein inheritance, and the additivity of zein gene expression in hybrids. Similarly, subtraction of a known (or suspected) parent from a hybrid gives a difference curve closely resembling that of the other parent, permitting determination or authentication of hybrid pedigrees.

CHROCP in its "automatic" mode may reveal types and amounts of varieties in grain mixtures (Figure 21). For example, elution times of gliadins from a mixture may permit one variety to be identified. When a chromatogram for gliadin of that variety is subtracted from that of the mixture, a difference curve resembling the other component of the mixture results. In addition, CHROCP estimates amounts of each component by adjusting the value of K to minimize negative deviations from a baseline, as expected for true samples. In the case shown in Figure 21, the computer predicted that B comprised 48% of the mixture; its known abundance was 50%. These preliminary results suggest that computer interpretation of RP-HPLC data has promise for analysis of real mixtures of cereal varieties or classes.
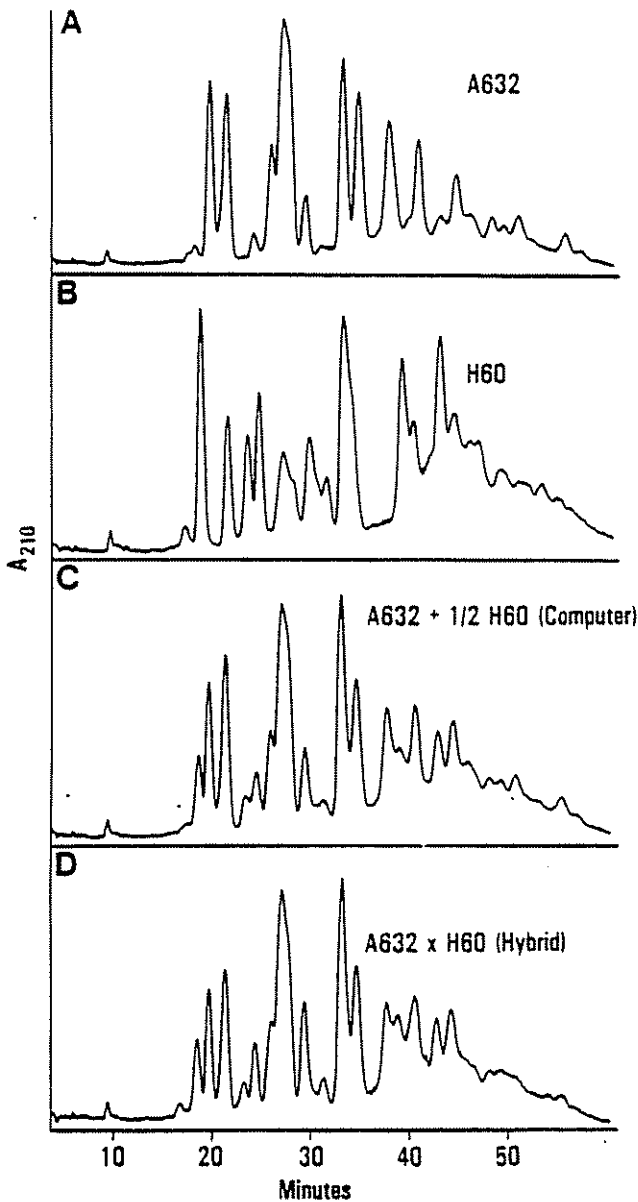
Figure 20. Computer comparison of maize inbred and hybrid lines. Chromatograms of inbreds A632 (A) and H60 (B) were added by computer program CHROCP in a 2:1 ratio, giving a computer-derived pattern (C). For comparison, the authentic hybrid A632× H60 is shown (D). Extracted zeins were chromatographed on SynChropak RP-P (C18) at 70° C, using a gradient of 48–58% acetonitrile (+ 0.1% trifluoroacetic acid) during 60 minutes. (Reprinted, with permission, from Bietz, 1986)
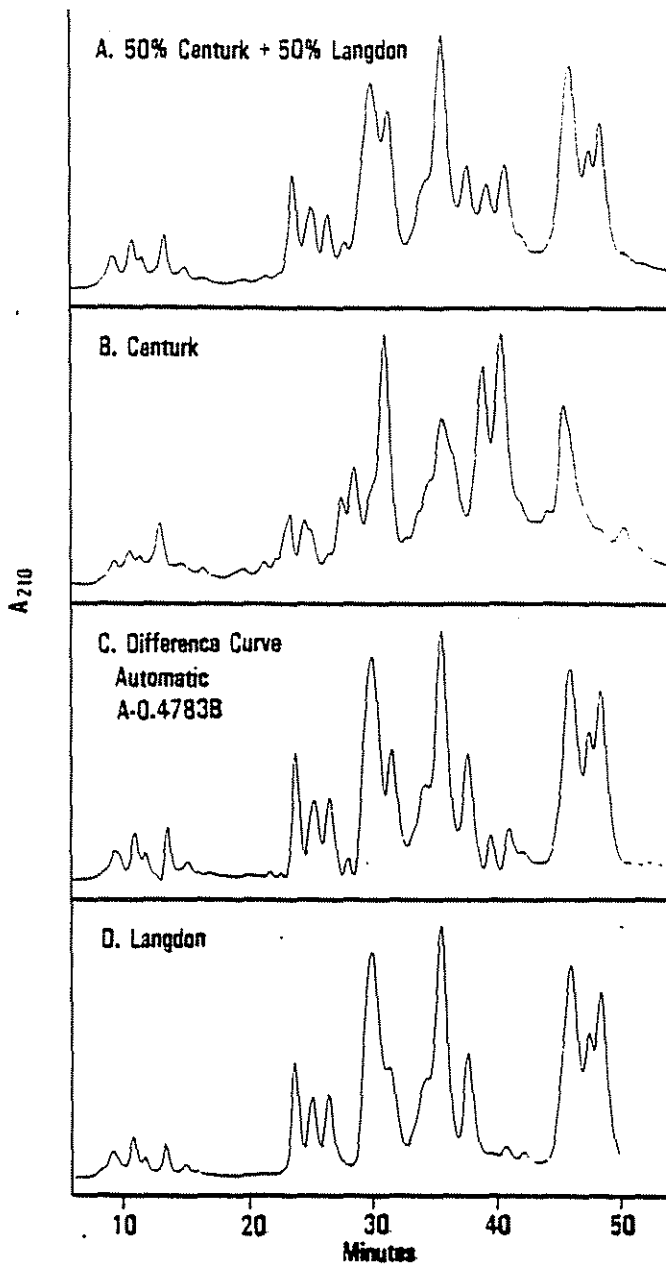
Figure 21. Computer analysis of mixed wheat by reversed-phase high-performance liquid chromatography of extracted gliadins. A chromatogram of Centurk gliadin (B) was subtracted from that of a synthetic (equal weight) mixture of the varieties Centurk and Langdon (A), using the automatic mode of program CHROCP, giving the difference curve C. Chromatogram D is of gliadin extracted from the other component of the mixture, Langdon wheat. Conditions were as in Figure 13. (Reprinted, with permission, from Bietz, 1986)

IV. SE-HPLC

A. Introduction

Size-exclusion chromatography (also called gel permeation or gel filtration) is useful for protein analysis and fractionation. Separations are based mainly on molecular size (although ionic adsorption may also occur). If proteins are larger than the support pores, they elute at the column's void volume; if they are small, they may freely penetrate pores and elute at the column's total liquid volume. Molecules of intermediate size elute between these limits at positions determined by their penetration of pores and their retardation by the column. Since proteins are sorted by size, molecular weight may be estimated from elution volumes.

Size-exclusion chromatography is one of the most useful methods for isolating and characterizing cereal proteins. For example, wheat gliadin can be fractionated on porous dextran gels such as Sephadex G-100 or G-200 into HMW gliadin, ω-gliadin, and LMW gliadin (see Figure 1 in Bietz and Wall, 1980), and subunits of glutenin can be divided into three fractions differing in molecular weight, solubility, and associative tendency (Huebner and Wall, 1974). HMW native glutenin may also be fractionated on porous agarose (Huebner and Wall, 1976), demonstrating that native glutenin's size distribution is related to breadmaking quality.

Conventional SE-chromatography has serious drawbacks, however. Most separations take a day or longer, and column beds are weak, necessitating low flow rates. Reproducibility is poor and accurate quantitation nearly impossible. Rigid SE-HPLC columns, therefore, seemed to have significant potential for the isolation and characterization of cereal proteins. These columns are physically and chemically stable, uniform, and inert, and they offer improved resolution because of small, uniform supports and uniform pores (Regnier and Gooding, 1980; Alfredson et al, 1982; Hearn et al, 1982; Wehr, 1984). Small samples may be automatically analyzed, and reproducibility and quantitation are excellent. The main advantage of SE-HPLC, however, is speed: separations that required days now take only 20–30 minutes. SE-HPLC is now becoming widely used for cereal protein analysis (Bietz, 1984a, 1985a). This section summarizes the conditions and techniques used and gives typical examples of SE-HPLC separations of cereal proteins.

B. Analytical Conditions and Methods

COLUMNS

Numerous silica-based SE-HPLC columns suitable for proteins are now available, and others useful under more diverse conditions or for larger proteins are becoming available (Hearn et al, 1982; Wehr, 1984). To date, most SE-HPLC studies of cereal proteins have used TSK-4000SW, TSK-3000SW, and TSK-2000SW columns (Toya-Soda), marketed by several suppliers. These columns are frequently larger ($\approx 7.5 \times 500$–600 mm) and more expensive than analytical RP-HPLC columns. They have pores of 450, 240, and 130 Å, respectively, making them useful in the molecular-weight range of 500 to 1 million (Alfredson et al, 1982). Guard columns (TSK-3000SW, 7.5×100 mm) are highly recommended to achieve reasonable column stability and lifetime; at

least hundreds of runs should be attainable. These columns do not, however, seem to last as long as RP-HPLC columns; once problems occur, lost resolution or increased pressure can seldom be reversed.

APPARATUS AND CONDITIONS

SE-HPLC systems are identical to those for RP-HPLC except that no gradients are required and only one pump is necessary since columns are eluted isocratically. Separations occur near ambient temperature, but constant temperature is recommended. Flow rates of 1.0 ml/min (200–300 psi) are common, but higher resolution at lower flow rates has been claimed. Samples of 1–10 μg of protein are usually analyzed (0.5–3.0 mg/ml). Samples are applied in a small volume (generally 10–50 μl) to minimize band spreading.

Nearly any solvent in the pH range 2–8 may be used in SE-HPLC; solvents containing denaturants (urea and guanidine hydrochloride) and detergents (SDS) are permissible. For analysis of cereal proteins (Bietz, 1984a), 0.1M sodium phosphate, pH 7.0, + 0.1% SDS, leads to an accurate relationship between molecular weight and elution volume. This solvent is essentially the same as that used in SDS-PAGE, which solubilizes all single-chained polypeptides and many complex HMW proteins.

PREPARATION OF SAMPLES

For fractionation of proteins by SE-HPLC in solvents that contain SDS, sample preparation is similar to that for SDS-PAGE (Bietz and Wall, 1972). Samples are incubated (overnight at room temperature, one to two hours at 50–60° C, or for a few minutes in a boiling water bath) with a solvent containing at least twice as much SDS (w/w) as protein. This ensures full complexing of SDS to proteins (generally 1.4 g/g). Adding urea (8M) to the sample-complexing solvent may facilitate the total denaturation of proteins, leading to the complete reduction of disulfide bonds (if a reducing agent is present) and complexing to SDS. The chromatographic solvent is generally used to dissolve samples to prevent baseline anomalies. A reducing agent (generally 1–5% ME) may be added to the complexing buffer to cleave disulfide bonds, or it may be omitted so that native proteins can be examined. Protein-SDS complexes do not dissociate significantly in the presence of SDS, so it is not necessary to include ME in SDS-containing solvents to prevent reoxidation. Proteins may be extracted directly from flour or meal under these conditions, or isolated proteins may be complexed to SDS for analysis. As in RP-HPLC, it is essential that all insoluble materials be removed from the samples; centrifugation is preferable for small samples, to avoid losses and because filters may adsorb some proteins.

STANDARDIZATION OF COLUMNS

Periodic analysis of standard proteins is recommended in SE-HPLC, as in RP-HPLC, to monitor resolution and the performance of the system. In addition, such analyses reveal the near-linear relationship between log molecular weight and the elution volume (or time) used to determine the molecular weight of sample proteins. Bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), chymotrypsinogen A (mol wt 25,700), cytochrome c (mol wt 11,700), and adenosine-5'-phosphate (to indicate total column volume) are useful and reliable standards under both reducing and nonreducing

conditions. For analyses of HMW native proteins on TSK-4000SW under nonreducing conditions, thyroglobulin (giving peaks corresponding to mol wts 660,000 and 330,000) and bovine serum albumin (mol wt 68,000 plus a mol wt 136,000 dimer) are also useful. Standards must be examined individually to determine homogeneity and elution volumes; subsequently, protein can be periodically used to monitor resolution and to update data for molecular weight determinations. Linear regression analysis is used to determine the slope and intercept in the determination of log molecular weight, which is subsequently used to estimate the sample's molecular weight from elution volumes (one should remember that the method's accuracy is only 5–10%). Computer programs are available that automatically determine molecular weight from retention times and estimate amounts of proteins in any molecular weight range. Computers can also reintegrate, replot, and compare data, as described above.

C. Examples and Applications

CHARACTERIZATION AND MOLECULAR WEIGHT DETERMINATION

SE-HPLC can quickly and reliably characterize and compare cereal proteins and estimate molecular weight. SE-HPLC of total gliadin (Figure 22A) reveals a major peak of mol wt ≈28,000 containing primarily α-, β-, and γ-gliadins. Smaller peaks of mol wt 11,000 (probably albumins or globulins), 41,000, and 63,000 (ω-gliadins) are also present, plus a peak of mol wt 105,000 (HMW gliadin). Resolution is similar to that of gel filtration (Bietz and Wall, 1980) of the same sample, and estimates of molecular weight agree with those from other techniques (Bietz and Wall, 1972). A purified γ-3 gliadin isolated by IEC (Huebner et al, 1967) was also revealed by SE-HPLC (Figure 22B) to be nearly homogeneous in molecular weight (43,000), which is consistent with estimates from amino acid analysis (Huebner et al, 1967) and SDS-PAGE (Bietz and Wall, 1972).

The resolution in Figure 22 is fairly good but certainly not remarkable; the resolution of SE-HPLC generally seems similar to that of conventional size-exclusion chromatography but inferior to that of SDS-PAGE. It is notable, however, that SE-HPLC characterizes samples and accurately determines molecular weight in 20–30 minutes, as compared to one to two days for gel filtration or SDS-PAGE.

Other SE-HPLC separations of wheat proteins described by Bietz (1984a) include glutenin subunits, for which results agree closely with other methods. SE-HPLC can also characterize the molecular weight of other cereal prolamins (Bietz, 1982), as well as total protein extracts from normal and yellow-berry kernels of triticale (Bietz and Sharma, 1983). In all cases, SE-HPLC results agree closely with those from other methods but are obtained automatically, rapidly, quantitatively, and at high sensitivity. These advantages are making SE-HPLC widely used for cereal protein characterization.

STUDY OF PROTEIN INTERACTIONS

SE-HPLC, like gel filtration, can examine association, aggregation, and disulfide bonding of cereal proteins with each other and with other endosperm constituents. An example is HMW gliadin, a heterogeneous oligomeric series

intermediate in molecular weight between gliadin and glutenin. HMW gliadin has a native molecular weight averaging 125,000 but dissociates to subunits of primarily mol wt 36,000 and 44,000 when its disulfide bonds are cleaved (Bietz and Wall, 1972, 1973, 1980). SE-HPLC can quickly and easily reveal this same
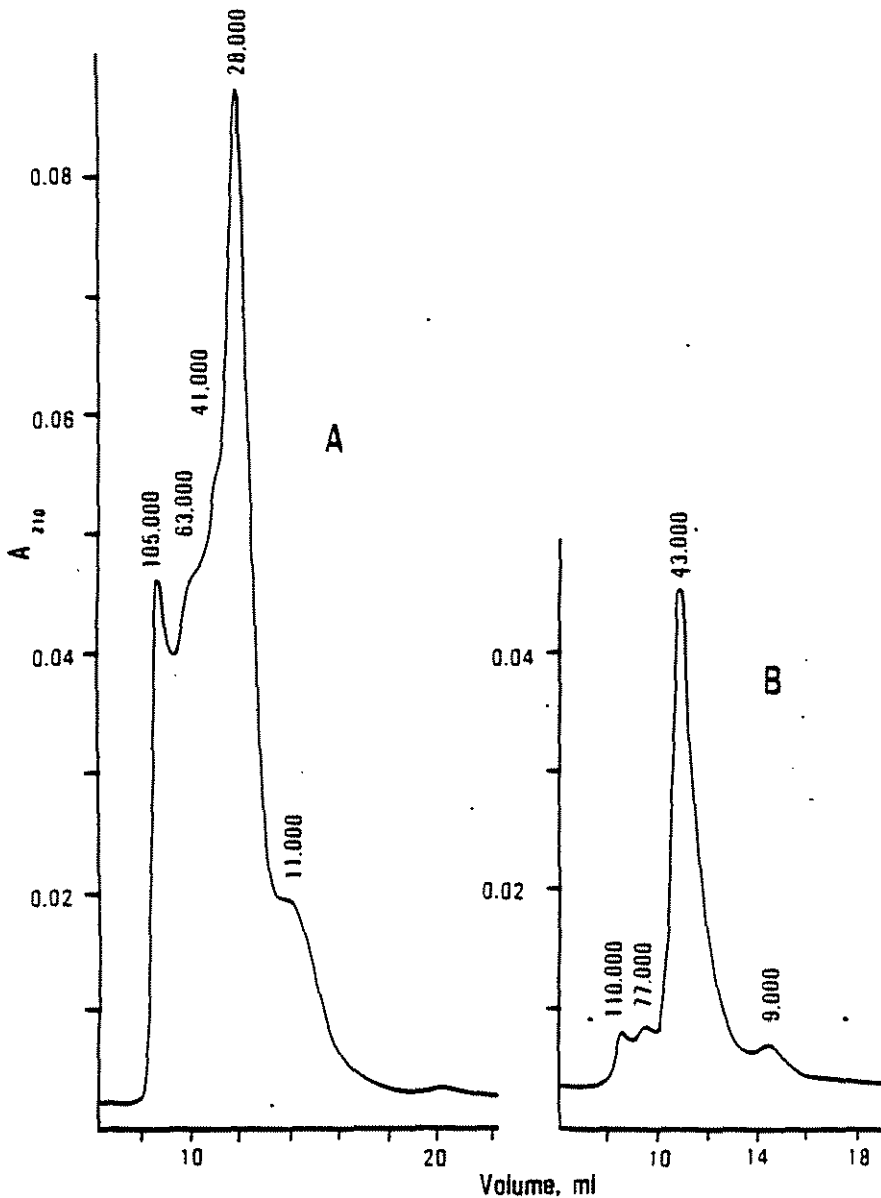

Figure 22. Comparison of crude and purified wheat gliadins by size-exclusion high-performance liquid chromatography on TSK-3000SW. A, Whole native Ponca gliadin; B, reduced γ-3 gliadin. Samples complexed to sodium dodecyl sulfate, in the absence of mercaptoethanol, were analyzed at 1.0 ml/min, and the column effluent was monitored at 210 nm. Estimated molecular weights are indicated for major peaks. (Reprinted, with permission, from Bietz, 1984a)

information: native HMW gliadin (Figure 23A) has a molecular weight primarily greater than 100,000; upon reduction, however (Figure 23B), molecular weights decrease dramatically (primarily to ≈41,000), revealing intermolecular disulfide bonds in the native protein.

Noncovalent interactions may also be revealed by SE-HPLC. If proteins are solubilized under various conditions or with denaturants or detergents that disrupt hydrogen, hydrophobic, or other noncovalent bonds, the associated or dissociated states of proteins may be observed and the types of bonding determined.

DIFFERENTIATION OF VARIETIES

Since significant molecular-weight differences related to quality occur among native glutenins of different varieties, analysis of these proteins by SE-HPLC would be highly desirable. One problem, however, is that native glutenin ranges up to mol wt 20,000,000 (Huebner and Wall, 1976), whereas the upper molecular-weight limit of TSK-4000SW is only one to two million.

It is possible, however, to analyze lower-molecular-weight native glutenin molecules by SE-HPLC, revealing information about genotype. Glutenins of four varieties were extracted with an SDS solution (without ME) after previous extraction of albumins, globulins, and gliadins. The glutenins were found to
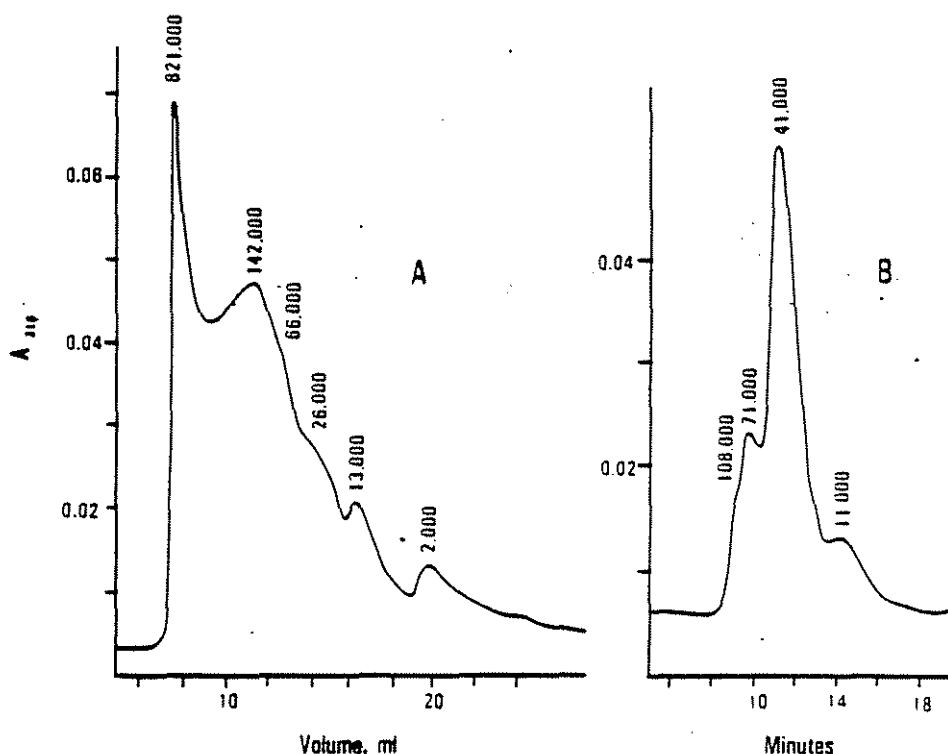

Figure 23. Comparison of native (A) and reduced (B) high-molecular-weight gliadin by size-exclusion high-performance liquid chromatography on TSK-4000SW. Other conditions were as in Figure 22. (Reprinted, with permission, from Bietz, 1984a)

differ markedly in SE-HPLC pattern (Figure 24) (Bietz, 1984a). Atlas 66, a high-protein soft red winter wheat used in breeding programs, had relatively little HMW glutenin in this extract but was rich in LMW proteins, which apparently incorporate noncovalently into glutenin. In contrast, Chinese Spring and Red Chief, wheats of poor baking quality, have large amounts of HMW protein. Centurk, a good bread wheat, has a balance of HMW and LMW species. Thus, SE-HPLC may differentiate genotypes by determining the molecular-weight distributions of HMW proteins, as well as predicting quality.

QUALITY PREDICTION BY SE-HPLC

In a further test of using SE-HPLC to estimate bread wheat quality, total protein extracts and SDS-soluble native glutenin from wheats varying in dough-mixing characteristics were fractionated on TSK-4000SW (see Figure 6 in Bietz, 1984a). More HMW protein was present in weak than in strong varieties.
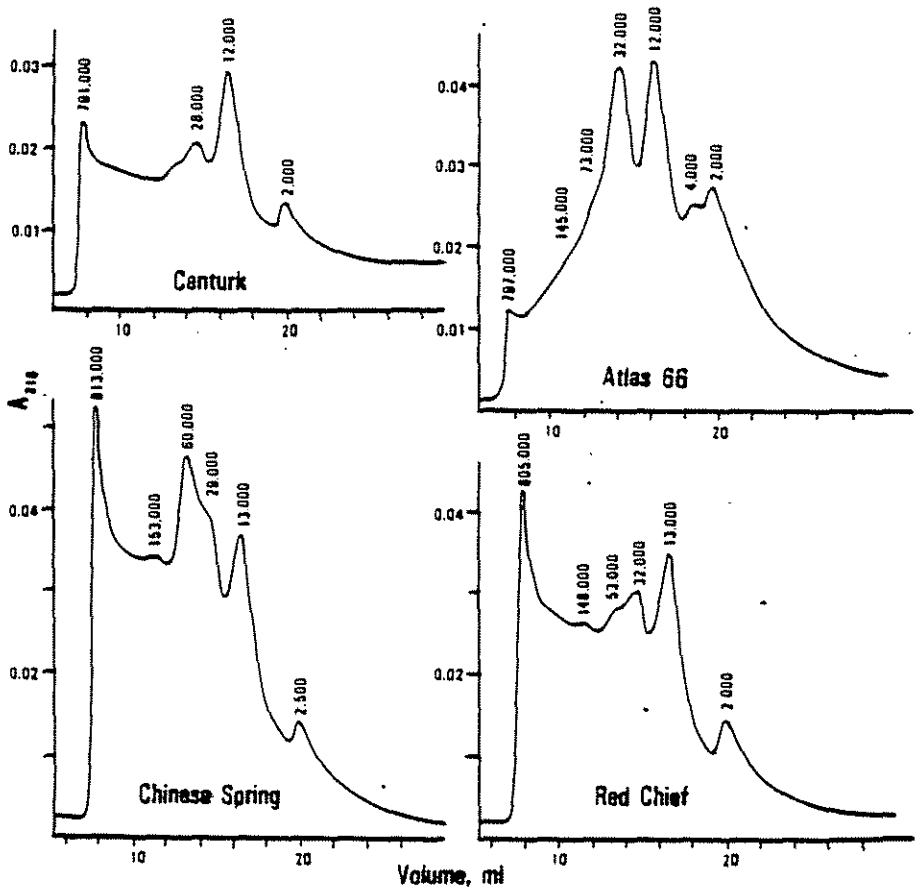

Figure 24. Comparison of native glutenin proteins, extracted with solvent containing sodium dodecyl sulfate, from single kernels of the wheat varieties Centurk, Atlas 66, Chinese Spring, and Red Chief by size-exclusion high-performance liquid chromatography on TSK-4000SW. Other conditions were as in Figure 22. (Reprinted, with permission, from Bietz, 1984a)

Further analyses showed native glutenin to be responsible for these results: glutenins from weak flours had more HMW protein than did glutenins from strong flours. These results agree with reports (Orth and Bushuk, 1972) that poor-quality wheats are rich in easily soluble glutenin, whereas good baking wheats have more residue protein, which is insoluble under mild extraction conditions. Thus, SE-HPLC confirms previous demonstrations that the molecular-weight distributions of flour proteins relate to breadmaking quality, and it suggests that rapid tests combining protein extraction with SE-HPLC should be able to predict quality.

Huebner and Bietz (1985) further examined the ability of SE-HPLC to predict dough-mixing time. Molecular-weight distributions of native proteins extracted with SDS solutions were related to dough-mixing time, but HMW glutenin was difficult to extract and was not sufficiently stable. Huebner and Bietz (1985) concluded that although SE-HPLC of HMW native wheat proteins can rapidly predict baking quality, further studies are necessary to improve the method's accuracy.

Huebner and Bietz (1985) also demonstrated that molecular-weight distributions of glutenin subunits, as determined by SE-HPLC, can be related to quality. After extraction of albumins, globulins, and gliadins, either HMW glutenin (residue protein) or total glutenin was reduced and alkylated, and subunits were analyzed on TSK-4000SW. Two distinct peaks resulted (see Figure 4 in Huebner and Bietz, 1985), corresponding to HMW and LMW glutenin subunits; relative amounts of these subunit types were significantly correlated with mixing time.

A final example of cereal quality prediction by SE-HPLC is its use to study changes in molecular weight of wheat proteins upon germination (sprouting). Kruger (1984) extracted proteins with neutral aqueous buffers from wheats germinated for different periods and examined molecular-weight distributions by SE-HPLC on TSK-3000SW. Results indicated that LMW peptides progressively form from proteins during germination and that the extent of such change is an indication of the approximate degree of germination of wheat. Kruger and Marchylo (1985b) subsequently used SE-HPLC to examine molecular-weight distributions of storage proteins of wheat, extracted with neutral phosphate buffer containing SDS, as a function of germination time. Total amounts of HMW proteins decreased slightly upon germination, whereas lower-molecular-weight peptides increased in amount. Such changes can predict the extent of germination, which substantially affects quality.

V. ION-EXCHANGE HPLC

Ion-exchange chromatography, primarily using cellulose columns, has long been one of the best available techniques for fractionating cereal proteins (e.g., Huebner and Wall, 1966; Charbonnier, 1974). Thus, silica-based IE-HPLC columns having equivalent or different functionalities should also be valuable for cereal protein isolation and characterization. Such columns should be more stable and uniform and have the potential for providing excellent resolution.

Numerous silica-based cation- and anion-exchange HPLC columns have been developed (Regnier and Gooding, 1980; Hearn et al, 1982; Wehr, 1984) and applied extensively to noncereal proteins. Attempts to apply some of these

columns to cereal endosperm storage proteins have had limited success (Bietz, 1985a; Ian Batey, personal communication). It is uncertain whether this is due to the unique characteristics of many cereal proteins or whether it reflects nonoptimal analytical conditions. It is likely, however, that once proper conditions are established or suitable columns are used, IE-HPLC will become highly valuable for cereal protein analysis.

One successful application of IE-HPLC to cereal proteins has been reported. Ion-exchange columns designated as "FPLC" (fast protein liquid chromatography) have now become available (Richey, 1982). The medium used to prepare such "MonoBead" columns is a composite hydrophilic polyether, modified to have anion exchange, cation exchange, or chromatofocusing functionality. Using Mono-Q (a strong anion-exchange column) for separation of negatively charged proteins, Batey (1984) achieved good separations of gliadins in about 20 minutes and demonstrated that most wheat varieties can be readily differentiated by the technique (Figure 25). Results were also found to be independent of environmental factors. To achieve these separations, Batey used
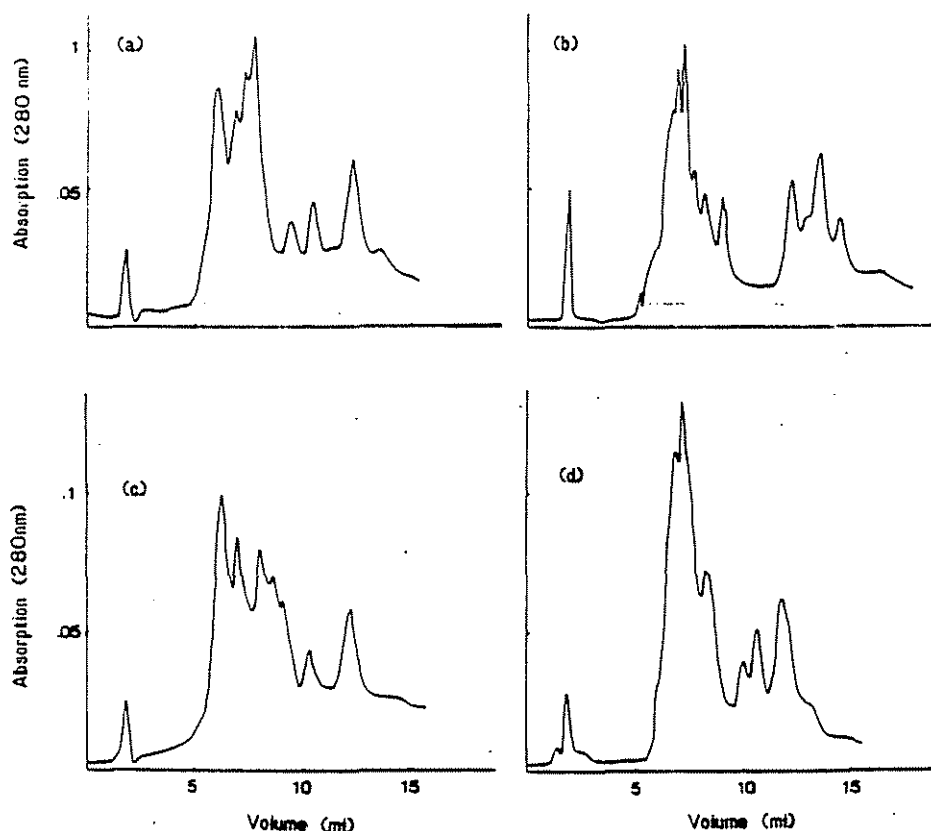

Figure 25. Elution patterns, obtained by fast protein liquid chromatography on a Mono-Q column at pH 10.4, of gliadins from the wheat cultivars Condor (a), Durati (b), Egret (c), and Eagle (d). Proteins were eluted with a gradient of 0–0.5M sodium acetate in a buffer containing 1M urea and 0.01M 3-(cyclohexylamino)-propane-sulphonic acid. (Reprinted, with permission, from Batey, 1984)

high-pH buffers (10.4 being optimal) because of the low amounts of glutamic and aspartic acid in gliadin and the positive charge of arginine except at high pH. The success of this method for gliadins may be due to the nonsilica nature of the column or to the relatively alkaline conditions used, which may emphasize charge differences among gliadins. Since many successful IEC separations of cereal proteins have been performed at relatively low pH using cation-exchange columns (e.g., Huebner and Wall, 1966; Charbonnier, 1974), however, it seems likely that cation-exchange HPLC using similar solvent conditions should provide separations superior to those already achieved on conventional supports, making IE-HPLC a widely used technique for cereal protein analysis.

VI. SUMMARY, CONCLUSIONS, AND FUTURE TRENDS

The wide applicability and suitability of HPLC for cereal protein analysis are now well established. Excellent instrumentation plus improved columns have combined to permit new and improved separations by RP-, SE-, and IE-HPLC. These methods have advantages in speed, sensitivity, reproducibility, resolution, automation, quantitation, recovery, and column stability. Both analytical and preparative separations are easily achieved, and RP-HPLC results complement those of other methods.

Although HPLC of cereal proteins has developed only recently, its application to specific problem areas has already been demonstrated. HPLC is, first of all, a valuable analytical (and preparative) technique for isolating and characterizing cereal proteins. It is highly useful to differentiate and identify varieties and genotypes by "fingerprinting" extracted proteins; it thus provides information useful for breeding, selection, and genetic improvement of all cereals. HPLC can also measure and predict cereal quality. HPLC is already being applied practically in some of these areas, and its use is bound to increase.

Improvements have already been realized in HPLC methods for analysis of cereal proteins, and more may be expected. New and improved columns, such as, for example, microbore columns, and better understanding of their optimal utilization will probably further improve resolution and speed. HPLC will become widely used for routine quality control in production, marketing, and utilization and for identification of genotypes in cereal grain breeding, as well as in research and development. Its use will further increase as new applications are discovered: for instance, computer analyses of HPLC data may indicate new predictors of cereal quality, leading to new analytical tests.

Thus, HPLC has become and will remain a powerful and indispensable method for isolation, characterization, and comparison of cereal proteins. HPLC techniques will permit better understanding, prediction, measurement, and improvement of functional and nutritional qualities and the assurance of good quality in all cereals and cereal-based products.

LITERATURE CITED

ALFREDSON, T. V., WEHR, C. T., TALLMAN, L., and KLINK, F. 1982. Evaluation of new microparticulate packings for aqueous steric exclusion chromatography. J. Liq. Chromatogr. 5:489-524.

BATEY, I. L. 1984. Wheat varietal identification by rapid ion-exchange chromatography of gliadins. J. Cereal Sci. 2:241-248.

BIETZ, J. A. 1979. Recent advances in the isolation and characterization of cereal proteins. Cereal Foods World 24:199-207.

BIETZ, J. A. 1982. Cereal prolamin evolution and homology revealed by sequence analysis. Biochem. Genet. 20:1039-1053.

BIETZ, J. A. 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. J. Chromatogr. 255:219-238.

BIETZ, J. A. 1984a. Analysis of wheat gluten proteins by high-performance liquid chromatography, part I. Bakers Dig. 58(1):15-17, 20-21, 32.

BIETZ, J. A. 1984b. Analysis of wheat gluten proteins by high-performance liquid chromatography, part II. Bakers Dig. 58(2):22, 24.

BIETZ, J. A. 1984c. Analysis of wheat gluten proteins by reversed-phase high-performance liquid chromatography: Techniques and applications. Pages 1-11 in: Proc. Int. Workshop Gluten Proteins, 2nd. A. Graveland and J. H. E. Moonen, eds. TNO, Wageningen, Netherlands.

BIETZ, J. A. 1984d. Report on HPLC analysis of various Arkan gliadins. In: Report of Workshop on Wheat Classing, March 8-9, 1984. Agric. Res. Serv., U.S. Dep. Agric., Beltsville, MD.

BIETZ, J. A. 1985a. High performance liquid chromatography: How proteins look in cereals. Cereal Chem. 62:201-212.

BIETZ, J. A. 1985b. Identification of maize inbreds and hybrids by reversed-phase high-performance liquid chromatography. (Abstr.) Cereal Foods World 30:562.

BIETZ, J. A. 1986. Reversed-phase high-performance liquid chromatography of cereal proteins: Methods and applications. In: Analytical Methods for Proteins. J. Cherry and R. Barford, eds. Am. Oil Chem. Soc., Champaign, IL. In press.

BIETZ, J. A., and BURNOUF, T. 1985. Chromosomal control of wheat gliadin: Analysis by reversed-phase high-performance liquid chromatography. Theor. Appl. Genet. 70:599-609.

BIETZ, J. A., and COBB, L. A. 1985. Improved procedures for rapid wheat varietal identification by reversed-phase high-performance liquid chromatography of gliadin. Cereal

Chem. 62:332-339.

BIETZ, J. A., and SHARMA, G. C. 1983. Differences in endosperm proteins between yellow berry and normal triticales. Crop Sci. 23:704-708.

BIETZ, J. A., and WALL, J. S. 1972. Wheat gluten subunits: Molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cereal Chem. 49:416-430.

BIETZ, J. A., and WALL, J. S. 1973. Isolation and characterization of gliadin-like subunits from glutenin. Cereal Chem. 50:537-547.

BIETZ, J. A., and WALL, J. S. 1980. Identity of high molecular weight gliadin and ethanol-soluble glutenin subunits of wheat: Relation to gluten structure. Cereal Chem. 57:415-421.

BIETZ, J. A., BURNOUF, T., COBB, L. A., and WALL, J. S. 1983. Differences in selectivity of large-pore reversed-phase high-performance liquid chromatography columns for analysis of cereal proteins. (Abstr.) Cereal Foods World 28:555-556.

BIETZ, J. A., BURNOUF, T., COBB, L. A., and WALL, J. S. 1984a. Gliadin analysis by reversed-phase high-performance liquid chromatography: Optimization of extraction conditions. Cereal Chem. 61:124-129.

BIETZ, J. A., BURNOUF, T., COBB, L. A., and WALL, J. S. 1984b. Wheat varietal identification and genetic analysis by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:129-135.

BRANLARD, G. 1983. Correlation between gliadin bands. Theor. Appl. Genet. 64:163-168.

BRIGHT, S. W. J., and SHEWRY, P. R. 1983. Improvement of protein quality in cereals. Crit. Rev. Plant Sci. 1:49-93.

BROWN, J. W. S., and FLAVELL, R. B. 1981. Fractionation of wheat gliadin and glutenin subunits by two-dimensional electrophoresis and the role of group 6 and group 2 chromosomes in gliadin synthesis. Theor. Appl. Genet. 59:349-359.

BURNOUF, T., and BIETZ, J. A. 1984a. Reversed-phase high-performance liquid chromatography of durum wheat gliadins: Relationships to durum wheat quality. J. Cereal Sci. 2:3-14.

BURNOUF, T., and BIETZ, J. A. 1984b. Reversed-phase high-performance liquid chromatography of reduced glutenin, a disulfide-bonded protein of wheat endosperm. J. Chromatogr. 299:185-199.

BURNOUF, T., and BIETZ, J. A. 1984c. Reversed-phase high-performance liquid chromatography of gluten proteins from aneuploid lines and related species of wheat.

(Abstr.) Cereal Foods World 29:506-507.

BURNOUF, T., and BIETZ, J. A. 1985. Chromosomal control of glutenin subunits in aneuploid lines of wheat: Analysis by reversed-phase high-performance liquid chromatography. Theor. Appl. Genet. 70:610-619.

BURNOUF, T., and BOURIQUET, R. 1980. Glutenin subunits of genetically related European hexaploid wheat cultivars: Their relation to bread-making quality. Theor. Appl. Genet. 58:107-111.

BURNOUF, T., BIETZ, J. A., COBB, L. A., and WALL, J. S. 1983. Reversed-phase high-performance liquid chromatography of gliadins from hexaploid wheat (*Triticum aestivum* L.) varieties cultivated in France and possibility of varietal identification. C.R. Hebd. Seances Acad. Sci. Ser. C 297:377-382.

BUSHUK, W., and ZILLMAN, R. R. 1978. Wheat cultivar identification by gliadin electrophoregrams. I. Apparatus, method and nomenclature. Can. J. Plant Sci. 58:505-515.

CALDWELL, K. A. 1979. The fractionation and purification of gliadins by hydrophobic interaction chromatography. J. Sci. Food Agric. 30:185-196.

CHARBONNIER, L. 1974. Isolation and characterization of ω-gliadin fractions. Biochim. Biophys. Acta 359:142-151.

CHRAMBACH, A., and RODBARD, D. 1971. Polyacrylamide gel electrophoresis. Science 172:440-451.

CHUNG, K. H., and POMERANZ, Y. 1979. Acid-soluble proteins of wheat flours. II. Binding to hydrophobic gels. Cereal Chem. 56:196-201.

COLE, E. W., TORRES, J. V., and KASARDA, D. D. 1983. Aggregation of A-gliadin: Gel permeation chromatography. Cereal Chem. 60:306-310.

DAL BELIN PERUFFO, A., and POGNA, N. E. 1982. Variations in the biotype composition of common wheat cultivars examined by gliadin electrophoresis. Cereal Res. Commun. 10:111-113.

DAMIDAUX, R., AUTRAN, J.-C., GRIGNAC, P., and FEILLET, P. 1978. Mise en évidence de relations applicables en sélection entre l'électrophoregramme des gliadines et les propriétés viscoélastiques du gluten de *Triticum durum* Desf. C.R. Hebd. Seances Acad. Sci. Ser. D 287:701-704.

ESEN, A., BIETZ, J. A., PAULIS, J. W., and WALL, J. S. 1982. Tandem repeats in the N-terminal sequence of a proline-rich protein from corn endosperm. Nature 296:678-679.

ESEN, A., BIETZ, J. A., PAULIS, J. W., and WALL, J. S. 1985. Isolation and characteri-

zation of a methionine-rich protein from maize endosperm. J. Cereal Sci. 3:143-152.

GOHEEN, S. C., and STEVENS, A. 1985. New techniques for separating proteins in hydrophobic interaction HPLC. BioTechniques 3:48-54.

HAGEN, G., and RUBENSTEIN, I. 1981. Complex organization of zein genes in maize. Gene 13:239-249.

HEARN, M. T. W., REGNIER, F. E., and WEHR, C. T. 1982. HPLC of peptides and proteins. Am. Lab. 14:18, 20, 23-24, 27-30, 35-36, 38-39.

HUEBNER, F. R., and BIETZ, J. A. 1984. Separation of wheat gliadins by preparative reversed-phase high-performance liquid chromatography. Cereal Chem. 61:544-549.

HUEBNER, F. R., and BIETZ, J. A. 1985. Detection of quality differences among wheats by high-performance liquid chromatography. J. Chromatogr. 327:333-342.

HUEBNER, F. R., and BIETZ, J. A. 1986. Bread baking quality of hard wheats determined by RP-HPLC of gliadins. J. Cereal Sci. In press.

HUEBNER, F. R., and WALL, J. S. 1966. Improved chromatographic separation of gliadin proteins on sulfoethyl cellulose. Cereal Chem. 43:325-335.

HUEBNER, F. R., and WALL, J. S. 1974. Wheat glutenin subunits. I. Preparative separation by gel-filtration and ion-exchange chromatography. Cereal Chem. 51:228-240.

HUEBNER, F. R., and WALL, J. S. 1976. Fractionation and quantitative differences of glutenin from wheat varieties varying in baking quality. Cereal Chem. 53:258-269.

HUEBNER, F. R., ROTHFUS, J. A., and WALL, J. S. 1967. Isolation and chemical comparison of different gamma-gliadins from hard red winter wheat flour. Cereal Chem. 44:222-229.

JACKSON, E. A., HOLT, L. M., and PAYNE, P. I. 1983. Characterisation of high-molecular-weight gliadin and low-molecular-weight glutenin subunits of wheat endosperm by two-dimensional electrophoresis and the chromosomal localisation of their controlling genes. Theor. Appl. Genet. 66:29-37.

JOHNSON, V. A., WILHELMI, K. D., KUHR, S. L., MATTERN, P. J., and SCHMIDT, J. W. 1978. Breeding progress for protein and lysine in wheat. Pages 825-835 in: Proc. Int. Wheat Genetics Symp, 5th. New Delhi. Indian Soc. Genetics Plant Breeding. Indian Agric. Res. Inst., New Delhi.

JONES, R. W., TAYLOR, N. W., and SENTI, F. R. 1959. Electrophoresis and fractionation of wheat gluten. Arch. Biochem. Biophys. 84:363-376.

JONES, B. L., LOOKHART, G. L., HALL, S. B., and FINNEY, K. F. 1982. Identification of wheat cultivars by gliadin electrophoresis: Electrophoregrams of the 88 wheat cultivars most commonly grown in the United States in 1979. Cereal Chem. 59:181-188.

JONES, B. L., LOOKHART, G. L., and JOHNSON, D. E. 1985. Improved separation and toxicity analysis methods for purothionins. Cereal Chem. 62:327-331.

KASARDA, D. D., BERNARDIN, J. E., and NIMMO, C. C. 1976. Wheat proteins. Pages 158-236 in: Advances in Cereal Science and Technology, Vol. 1. Y. Pomeranz, ed. Am. Assoc. Cereal Chem., St, Paul, MN.

KIKTA, E. J., Jr., and STANGE, A. E. 1977. Phenones: A family of compounds broadly applicable to use as internal standards in high-performance liquid chromatography. Application to the analysis of carbofuran. J. Chromatogr. 138:41-45.

KRUGER, J. E. 1984. Rapid analysis of changes in the molecular weight distribution of buffer-soluble proteins during germination of wheat. Cereal Chem. 61:205-208.

KRUGER, J. E., and MARCHYLO, B. A. 1985a. Selection of column and operating conditions for reversed-phase high-performance liquid chromatography of proteins in Canadian wheat. Can. J. Plant Sci. 65:285-298.

KRUGER, J. E., and MARCHYLO, B. A. 1985b. Examination of the mobilization of storage proteins of wheat kernels during germination by high-performance reversed-phase and gel permeation chromatography. Cereal Chem. 62:1-5.

LOOKHART, G. L. 1985a. Identification of oat cultivars by combining polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography. Cereal Chem. 62:345-350.

LOOKHART, G. L. 1985b. Gliadin electrophoretic variations in foundation Arkan grown at 16 Kansas agricultural experiment stations in 1983. Cereal Chem. 62:355-360.

LOOKHART, G. L., and POMERANZ, Y. 1985a. Characterization of oat species by polyacrylamide gel electrophoresis and high-performance liquid chromatography of their prolamin proteins. Cereal Chem. 62:162-166.

LOOKHART, G. L., and POMERANZ, Y. 1985b. Gliadin high-performance liquid chromatography and polyacrylamide gel electrophoresis patterns of wheats grown with fertilizer treatments in the United States and Australia on sulfur-deficient soils. Cereal Chem. 62:227-229.

LOOKHART, G. L., JONES, B. L., WALKER, D. E., HALL, S. B., and COOPER, D. B. 1983. Computer-assisted method for identifying wheat cultivars from their gliadin electrophoregrams. Cereal Chem. 60:111-115.

LOOKHART, G. L., LAI, F. S., and POMERANZ, Y. 1985. Variability in gliadin electrophoregrams and hardness of individual wheat kernels selected from foundation seed on the basis of grain morphology. Cereal Chem. 62:185-190.

MARCHYLO, B. A., and KRUGER, J. E. 1984. Identification of Canadian barley cultivars by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:295-301.

MARCHYLO, B. A., and KRUGER, J. E. 1985. Assessment of RP-HPLC columns to separate hordein proteins and identify cultivars of barley and barley malt. J. Am. Soc. Brew. Chem. 43:29-35.

MARTIN, T. J., BOCKUS, W. W., BROWDER, L. E., FINNEY, K. F., HATCHETT, J. H., and WETZEL, D. L. 1983. Registration of Arkan wheat. Crop Sci. 23:1221-1222.

ORTH, R. A., and BUSHUK, W. 1972. A comparative study of the proteins of wheats of diverse baking qualities. Cereal Chem. 49:268-275.

OSBORNE, T. B. 1907. The proteins of the wheat kernel. Carnegie Institute Washington, Washington, DC.

PAULIS, J. W., and BIETZ, J. A. 1985. Fractionation and characterization of zein polypeptides from corn endosperm by high-performance liquid chromatography. (Abstr. 48) Book of Abstracts. Am. Chem. Soc. Div. of Agric. and Food Chem., 189th ACS Natl. Meet. April 28-May 3. Am. Chem. Soc., Washington, DC.

PAULIS, J. W., and BIETZ, J. A. 1986. Separation of alcohol-soluble corn proteins by reversed-phase high-performance liquid chromatography. J. Cereal Sci. In press.

PAULIS, J. W., WALL, J. S., and KWOLEK, W. F. 1974. A rapid turbidimetric analysis for zein in corn and its correlation with lysine content. J. Agric. Food Chem. 22:313-317.

PAULIS, J. W., BIETZ, J. A., and WALL, J. S. 1975. Corn protein subunits: Molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Agric. Food Chem. 23:197-201.

PAYNE, P. I., and LAWRENCE, G. J. 1983. Catalogue of alleles for the complex gene loci, *Glu-A1*, *Glu-B1*, and *Glu-D1* which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res. Commun. 11:29-35.

PAYNE, P. I., and RHODES, A. P. 1983. Cereal storage proteins: Structure and role in agriculture and food technology. Pages 346-369 in: Encyclopedia of Plant Physiology

14A. Springer-Verlag, Berlin.

PAYNE, P. I., HOLT, L. M., and LAW, C. N. 1981. Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. I. Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theor. Appl. Genet. 60:229-236.

PAYNE, P. I., HOLT, L. M., JACKSON, E. A., and LAW, C. N. 1984. Wheat storage proteins: Their genetics and their potential for manipulation by plant breeding. Philos. Trans. R. Soc. London Ser. B 304:359-371.

POPINEAU, Y., and GODON, B. 1978. Application de la chromatographie d'interaction hydrophobe au fractionnement des prolamines du blé tendre. C.R. Hebd. Seances Acad. Sci. Ser. D 287:1051-1054.

REGNIER, F. E., and GOODING, K. M. 1980. High-performance liquid chromatography of proteins. Anal. Biochem. 103:1-25.

RICHEY, J. 1982. FPLC: A comprehensive separation technique for biopolymers. Am. Lab. 14:104, 107-110, 112-114, 116-119, 121-124, 126-129.

SAPIRSTEIN, H. D., and BUSHUK, W. 1985. Computer-assisted analysis of gliadin electrophoregrams. II. Wheat cultivar identification and class comparisons. Cereal Chem. 62:377-392.

SASTRY, L. V. S., PAULIS, J. W., BIETZ, J. A., and WALL, J. S. 1986a. Genetic variation of storage proteins in sorghum grain: Studies by isoelectric focusing and high-performance liquid chromatography. Cereal Chem. In press.

SASTRY, L. V. S., PAULIS, J. W., COBB, L. A., WALL, J. S., and AXTELL, J. D. 1986b. Inheritance of storage proteins in high lysine sorghums. J. Agric. Food Chem. In press.

SCHOFIELD, J. D., BOTTOMLEY, R. C., TIMMS, M. F., and BOOTH, M. R. 1983. The effect of heat on wheat gluten and the involvement of sulphydryl-disulphide interchange reactions. J. Cereal Sci. 1:241-253.

SHEWRY, P. R., MIFLIN, B. J., and KASARDA, D. D. 1984. The structural and evolutionary relationships of the prolamin storage proteins of barley, rye and wheat. Philos. Trans. R. Soc. London Ser. B 304:297-308.

SOZINOV, A. A., and POPERELYA, F. A. 1980. Genetic classification of prolamines and its use for plant breeding. Ann. Technol. Agric. 29:229-245.

SUMMERS, K. A., KIRLEIS, A. W., and NIELSEN, N. C. 1983. Characterization of sorghum prolamins. (Abstr. 163) Plant Physiol. (Suppl.) 72:30.

TANFORD, C. 1970. Protein denaturation. Part C. Theoretical models for the mechanism of denaturation. Adv. Protein Chem. 24:1-95.

WALL, J. S. 1979. The role of wheat proteins in determining baking quality. Page 275-311 in: Recent Advances in the Biochemistry of Cereals. D. L. Laidman and R. G. Wyn Jones, eds. Academic Press, London.

WALL, J. S., and PAULIS, J. W. 1978. Corn and sorghum grain proteins. Pages 135-219 in: Advances in Cereal Science and Technology, Vol. 2. Y. Pomeranz, ed. Am. Assoc. Cereal Chem., St. Paul, MN.

WALL, J. S., FEY, D. A., PAULIS, J. W., and LANDRY, J. 1984. Improved two-dimensional electrophoretic separation of zein proteins: Application to study of zein inheritance in corn genotypes. Cereal Chem. 61:141-146.

WEHR, C. T. 1984. Commercially available HPLC columns for chromatography of proteins, peptides, and amino acids. Page 31 in: Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins, Vol. I. W. S. Hancock, ed. CRC Press, Inc., Boca Raton, FL.

WILSON, C. M. 1984. Isoelectric focusing of zein in agarose. Cereal Chem. 61:198-200.

WOYCHIK, J. H., BOUNDY, J. A., and DIMLER, R. J. 1961. Starch gel electrophoresis of wheat gluten proteins with concentrated urea. Arch. Biochem. Biophys. 94:477-482.

WRIGLEY, C. W. 1977. Characterisation and analysis of cereal products in foods by protein electrophoresis. Food Technol. Aust. 29:17-20.

WRIGLEY, C. W. 1980. The genetic and chemical significance of varietal differences in gluten composition. Ann. Technol. Agric. 29:213-227.

WRIGLEY, C. W., and SHEPHERD, K. W. 1973. Electrofocusing of grain proteins from wheat genotypes. Ann. N.Y. Acad. Sci. 209:154-162.

WRIGLEY, C. W., AUTRAN, J.-C., and BUSHUK, W. 1982. Identification of cereal varieties by gel electrophoresis of the grain proteins. Pages 211-259 in: Advances in Cereal Science and Technology, Vol. 5. Y. Pomeranz, ed. Am. Assoc. Cereal Chem., St. Paul, MN.

WRIGLEY, C. W., DU CROS, D. L., FULLINGTON, J. G., and KASARDA, D. D. 1984. Changes in polypeptide composition and grain quality due to sulfur deficiency in wheat. J. Cereal Sci. 2:15-24.

ZAWISTOWSKA, U., BIETZ, J. A., and BUSHUK, W. 1985. Low-molecular weight wheat proteins with high affinity for flour lipids. (Abstr.) Cereal Foods World 30:544.